NUCLEIC ACID AMPLIFICATION FROM INDIVIDUAL CELLS

Laser Capture Microdissection

Mammalian tissues are histologically and biologically heterogeneous, and typically contain multiple cellular components, such as epithelial, mesenchymal (i.e., stromal), and inflammatory cells. Laser capture microdissection (LCM) offers a rapid and precise method of isolating and removing specified cells from complex tissues for subsequent analysis of their RNA, DNA, or protein content, thereby allowing assessment of the role of the cell type in the normal physiologic or disease process being studied. LCM has been utilized to study molecular changes during the neoplastic progression of specific cell types (Sgroi et al., 1999; Paweletz et al., 2000), and to understand the role of particular cell types in normal organ function (Glasow et al., 1998; Jin et al., 1999) and in various disease processes (Fend et al., 1999a; Sawyer et al., 2000). LCM has the potential to contribute to the understanding of many cellular processes, particularly processes involving multiple cell types, such as embryonic development, tissue differentiation and function, aging, and disease.

There are methods for tissue microdissection other than LCM, such as laser microbeam microdissection and laser-pressure catapulting, in which a fine laser beam is used to cut around individual or groups of cells and then laser energy is used to "catapult" the cells out of the tissue section and allow their collection (P.A.L.M. Mikrolaser Technologie; *http://www.palm-mikrolaser.com*); however, currently, Arcturus Engineering is the only manufacturer of instrumentation for LCM. Arcturus Engineering (*http://www.arctur.com*) can be contacted for details about the various LCM systems available and the current prices of instrumentation and consumables.

In this unit, protocols for the preparation of mammalian frozen tissues (see Basic Protocol 1), fixed tissues (see Basic Protocol 2), and cytologic specimens (see Basic Protocols 3 and 4) for LCM, including hematoxylin and eosin staining (H&E; see Basic Protocol 5 and *UNIT 14.5*), are presented, as well as a protocol for the performance of LCM utilizing the PixCell I or II Laser Capture Microdissection System manufactured by Arcturus Engineering (see Basic Protocol 6). Also provided is a protocol for tissue processing and paraffin embedding (see Support Protocol), and recipes for lysis buffers for the recovery of nucleic acids and proteins (see Reagents and Solutions). The Commentary section addresses the types of specimens that can be utilized for LCM and approaches to staining of specimens for cell visualization (see Critical Parameters). Emphasis is placed on the preparation of tissue or cytologic specimens as this is critical to effective LCM. Resources available on-line are given at the end of the unit (see Internet Resources).

PREPARATION OF FROZEN SECTIONS FOR LCM

BASIC PROTOCOL 1

Embedding and freezing is a way to preserve specimens and stabilize them for long-term storage and sectioning (also see *UNIT 14.2*). Tissue is embedded in a viscous compound, such as optimal cutting temperature (OCT; Tissue-Tek) medium, and rapidly frozen on dry ice. For long-term storage (i.e., months to years), liquid nitrogen offers the best preservation of protein and RNA. Storage at −80°C is adequate for shorter time periods (i.e., a few days to several weeks).

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Materials

Embedding medium (e.g., OCT; Tissue-Tek)
~1-cm maximum-dimension tissue samples

Cryomolds (Tissue-Tek)
Dry-ice container with lid
Aluminum foil
Microm cryostat, refrigerated to −20°C with tissue platform (chuck) and appropriate blades (Richard-Allan Scientific)
Glass slides (e.g., Gold Seal plain uncoated slides; Becton Dickinson)
No. 2 pencil or slide marker
Slide boxes (optional)

Embed tissue

1. Place a labeled empty cryomold on dry ice in a container for 1 min. Keep on dry ice during the entire embedding procedure.
2. Cover the bottom of the cryomold with ~2 to 3 mm embedding medium.
3. Place the tissue to be frozen against the bottom of the cryomold in the medium before it hardens (this may take <1 min depending on the amount of OCT used).

   To facilitate cutting, the tissue should be relatively small (i.e., 1 cm in maximum dimension) and the desired cutting surface should be flush against the bottom.

4. Fill the cryomold containing the base of embedding medium and frozen tissue with more embedding medium. Cover the dry ice container and allow the embedding medium to harden (several minutes).

   The medium will turn from translucent to white when frozen.

5. Wrap the resulting tissue block, still in the cryomold, in aluminum foil and keep in a −80°C freezer or in liquid nitrogen until cutting.

   Tissue for RNA extraction should be frozen as quickly as possible after resection. The method described here is preferred for LCM because tissue processed in this manner is more amenable to cryostat sectioning and offers acceptable histomorphology. More rapid methods of freezing tissue, such as direct immersion into liquid nitrogen, isopentane chilled to −160°C (Sheehan and Hrapchak, 1987a), or the vapor phase of liquid nitrogen, can also be utilized; however, these methods are more technically difficult when incorporating cryostat embedding media and are more likely to result in cracking of the tissue block. Tissues that were rapidly frozen without embedding medium can be postembedded in cryostat embedding medium, but will thaw somewhat in the process. This can compromise RNA preservation and introduce undesirable histologic artifacts.

Section tissue

6. Remove the tissue block from the cryomold and attach it to the tissue platform (chuck) in the cryostat, with additional embedding media serving as the "glue" at the interface. Apply just enough embedding media to cover the surface of the chuck and quickly attach the frozen tissue block before the "glue" hardens completely.

   The cutting surface should be as parallel as possible to the chuck surface.

7. Allow the block to equilibrate to the cryostat temperature (i.e., −20°C) ≥15 min.
8. Cut 5- to 10-μm sections onto glass slides that have been sitting at room temperature and previously labeled with identifying numbers and or letters using a no. 2 pencil or a permanent marker designed for labeling slides.

Glass slides can be plain uncoated, charged, or silanized. The properties of glass slides that allow tissue adherence are variable among different brands, even with plain uncoated slides. It is important to use slides that allow tissue sections to adhere well enough that they do not fall off during staining, but not so tightly that the tissue cannot be captured. It is likely that different brands and types of slides will have to be tried, and that slides used successfully for formalin-fixed paraffin-embedded sections may not be optimal for frozen ones. The authors have found the Becton Dickinson Gold Seal plain uncoated slides work well for LCM of frozen sections in their laboratory. It is best to begin with plain uncoated slides, and if tissue sections do not adhere well enough to allow staining, to try charged or silanized slides. Adhesives, such as Sta-On (Surgipath) can be applied directly to the slides or gelatin, or can be added to the water bath during histologic sectioning; however, these may limit the transfer efficiency of LCM.

It is important to mount the tissue as close to the center of the slide as possible. If the tissue is too far off center, the slide cannot be positioned so that the vacuum slide holder can function during microdissection.

If sections are particularly friable and thus difficult to cut, the tissue may be too cold; therefore, the time allowed for the block to equilibrate to −20°C may need to be extended. Sections should be without folds and lie as flat as possible on the slides.

Sections with >10-µm thickness are difficult to visualize. The authors prefer sections of 5- to 6-µm thickness. Thicker sections will require a larger spot size and therefore a higher laser-energy level.

9. Keep the slides in the cryostat or on dry ice if LCM is to be performed that day. Alternatively, store in slide boxes at −80°C until needed.

   The duration of preservation of RNA and protein in frozen sections at −80°C is not well documented and likely depends on the tissue and the desired analyte. Although storage over several weeks or even months at −80°C may preserve the analyte of interest well, if this has not been assessed, we recommend limiting storage of frozen sections prior to microdissection to one week.

10. Stain slides (see Basic Protocol 5) just prior to LCM.

    IMPORTANT NOTE: *Do not allow the slides to dry or thaw at room temperature prior to staining and dehydration. This is critical for successful LCM. Drying and thawing causes the tissue to adhere tightly to the slide and will decrease the transfer efficiency of LCM. Additionally, it may contribute to the degradation of RNA.*

PREPARATION OF FIXED PARAFFIN-EMBEDDED SECTIONS

BASIC PROTOCOL 2

Paraffin embedding is a process in which fixed tissue—utilizing neutral buffered formalin (NBF) or another fixative—is infiltrated and then placed into liquefied paraffin to stabilize it for long-term storage and easy sectioning (*UNIT 14.1*). While fixation is performed to preserve the morphology of the tissue for histologic examination, it also effects the DNA, RNA, and protein content. Formalin fixation is the standard for morphologic preservation of tissue and has been used by most pathology laboratories for decades; however, it creates cross-links between nucleic acids and proteins, and between different proteins. This cross-linking interferes with recovery of DNA, RNA, and proteins from fixed tissue, as well as the amplification of DNA and RNA by PCR (Arnold et al., 1996; Coombs et al., 1999; Goldsworthy et al., 1999; Masuda et al., 1999); however, short lengths of DNA, up to ~200 bp, can be reliably amplified after extraction from formalin-fixed paraffin-embedded (FFPE) tissue. RNA is a more labile species, and formalin fixation and paraffin embedding greatly interfere with its recovery. Attempts to break cross-links and thereby improve recovery of nucleic acids and protein have been utilized with varying degrees of success (Ikeda et al., 1998; Coombs et al., 1999; Masuda et al., 1999). Optimization and standardization of methods to break the cross-links caused by formalin fixation is a goal

of many researchers. Studies have shown that, among commonly used fixatives, formalin has the worst effects on RNA, while ethanol (i.e., 70% or 95% ethanol) or ethanol-based fixatives, available from suppliers of histology-related materials (e.g., Richard-Allan Scientific), offer the best RNA preservation (Goldsworthy et al., 1999; Shibutani et al., 2000).

In this protocol, it is assumed that most researchers will procure fixed and embedded tissue from pathology laboratories or other sources and may have no control over fixation and processing of tissues; however, a suggested protocol for fixation and tissue processing (see Support Protocol) has been included in the event the researcher is prospectively collecting human or animal tissues and has some degree of control over these processes.

Materials

Paraffin-embedded tissue block mounted on appropriate microtome chuck (see Support Protocol)
Xylene
100%, 95%, and 70% ethanol

Microtome and microtome blades (disposable preferred; Richard-Allan Scientific), clean
43° to 44°C water bath
Histologic slides, plain uncoated, charged, or silanized
37° to 42°C oven (optional)
Coplin jars or other solvent containers

Section tissue

1. Cut 5- to 10-µm sections of a paraffin-embedded tissue block mounted on an appropriate chuck on a clean microtome with a clean blade.

   IMPORTANT NOTE: *Careful attention should be given during sectioning and mounting of paraffin-embedded tissue to prevent carryover. Carryover contamination of one specimen from another or transfer of material from one region of a section to another can lead to spurious results. The microtome used to cut sections should be kept clean and excess paraffin and tissue fragments should be wiped from the area with a simple gauze pad. A fresh microtome blade should be used for each block and disposable blades used if possible.*

   Sections of 5-µm thickness are optimal for LCM, but the thickness should be dependent on the size of the cells to be microdissected.

2. Float resulting paraffin ribbons on 43° to 44°C deionized water in a water bath to smooth out and eliminate folds and wrinkles.

   The water should be changed frequently to avoid contamination of sections by tissue fragments from other tissues and to minimize growth of environmental microorganisms. The authors currently do not recommend using formalin-fixed paraffin-embedded tissue for RNA analysis; however, the authors and others have successfully performed RT-PCR on alcohol-fixed paraffin-embedded tissues. If sections will be microdissected for RNA, consideration should be given to using RNase-free water (UNIT 4.1).

   Some histopathology laboratories use an adhesive in the water bath to better adhere the tissue section to the slide. As this may result in reduced LCM transfer of tissue, it is not recommended.

3. Mount sections on histologic glass slides.

   Clean uncoated plain, charged, or silanized histological slides can be used. The authors have successfully performed LCM utilizing many brands of uncoated glass slides, as well as charged slides, with fixed and paraffin-embedded tissues.

   It is important to mount the tissue as close to the center of the slide as possible. If the tissue is too far off center, the slide cannot be positioned so that the vacuum slide holder can function during microdissection.

4. Air dry the paraffinized sections overnight or bake up to 8 hr at 37° to 42°C.

As with frozen sections, the desired result is for the tissue to remain adherent to the slide during staining, but not be so adherent as to prevent tissue transfer by LCM. Baking the slides will cause the sections to be more adherent than air drying. Relevant variables that affect LCM include the type of slide, whether the sample is air dried or baked, the duration of baking, and the type of tissue being microdissected.

Remove paraffin

5. Allow the slide containing the tissue section to remain in the following solutions, in Coplin jars or other solvent containers, for the specified times in the specified order:

Xylene	5 min
Xylene	5 min
100% ethanol	30 sec
95% ethanol	30 sec
70% ethanol	30 sec

In order to proceed with histologic staining and LCM following sectioning, paraffin must be removed from the tissue sections.

If RNA is to be analyzed, consideration should be given to preparing the 95% and 70% ethanol solutions with RNase-free water. The authors routinely utilize sterile or distilled water and typically achieve good RNA recovery.

6. Proceed with hematoxylin and eosin staining (see Basic Protocol 5).

PREPARATION OF CYTOLOGIC SPECIMENS FOR LCM: DIRECT SMEARS

BASIC PROTOCOL 3

Cellular elements in body fluids or fine-needle aspirates and cultured cells do not readily lend themselves to sectioning, but can easily be prepared for LCM by making direct smears or cytospin preparations. The choice as to which to use will depend upon the anticipated cellularity of the sample. Highly cellular samples can be easily and rapidly prepared as direct smears and effectively utilized for LCM, whereas less cellular samples are better concentrated and prepared as cytospin preparations. To determine if the sample requires concentration, make a direct smear as described below and examine it under the microscope. If the concentration of cells is such that the desired number of cells for LCM can be located in 1 to 4 areas each with a diameter of 0.5 cm (the appropriate diameter of the "cap" used to capture the cells of interest during LCM), the specimen does not require concentration. If however, the concentration of cells is so low that the number of desired cells is not present or the cells are so widely spaced that it will require five or more caps to obtain them, specimen concentration is recommended. For specimens contaminated with undesired blood elements (i.e., red blood cells or white cells that are not intended to be microdissected), use the protocol for cytologic smears or cytospins containing excessive blood as the contaminant (see Alternate Protocol 1). The same basic caveats apply to cytologic specimens as histologic sections—i.e., ethanol is the preferred fixative (especially for RNA analysis), the cells should never be allowed to dry on the slide prior to fixation, and the fixed and stained cells should be adequately dehydrated prior to LCM.

Materials

High-cellularity sample: cellular fluid (e.g., fine-needle aspiration, suspended cultured cells) or fresh tissue
95% ethanol
Hemocytometer cover (optional)
Glass slides, clean
Scalpel blade (fresh tissue)

1a. *For cellular fluid:* Place a drop of cellular fluid (i.e., fine-needle aspiration samples or cultured cells suspended in medium), no larger than 5 mm in diameter, towards the label end of a clean glass slide. Quickly utilize the edge of another glass slide, or preferably a hemacytometer cover, to thinly spread the drop (i.e., as if making a blood-smear preparation) on the slide in a single motion, relying on capillary action between the liquid and the two slides to spread the liquid in a uniform, thin-layer across the length and width of the slide. Do not apply excessive force which might result in crushing or shearing of cells.

Plain uncoated, charged, or silanized glass slides can be used.

We prefer to prepare cytologic smears with a hemocytometer cover because its width is slightly less than that of the standard glass microscopic slide and the resulting smear (i.e., cells) is not spread to, or off, the edge of the slide.

1b. *For fresh tissue:* Quickly sample by scraping tissue with a scalpel blade and then rapidly spread the scraped sample on a glass slide with the blade.

This is a quick and useful method of specimen preparation for tissues in which the desired cells can be readily identified cytologically, such as highly malignant cells.

2. Immediately after spreading, immerse the smear in 95% ethanol without allowing it to dry. Incubate 10 min.
3. Transfer to 70% ethanol for 30 sec.
4. Proceed to hematoxylin and eosin staining (see Basic Protocol 5).

BASIC PROTOCOL 4

PREPARATION OF CYTOLOGIC SPECIMENS FOR LCM: CYTOSPIN METHOD

Cytospin preparations can be used for any cytologic sample but are preferred for samples of low cellularity. Cytospin instrumentation allows cellular fluids to be simultaneously concentrated and placed on a glass slide. Using centrifugation, these instruments spin cell suspensions onto a microscope slide as the suspension medium is simultaneously absorbed by a blotter. The result is a monolayer of well-preserved well-displayed cells within a 6-mm^2 area on the slide. Another alternative for samples of low cellularity is to centrifuge the sample, decant the supernatant, and make a direct smear (see Basic Protocol 3) from the sediment. Particularly bloody specimens may benefit from the protocol provided below (see Alternate Protocol 1). To avoid RNA, DNA, or protein degradation, the cytologic samples should be processed and fixed in 95% ethanol shortly after collection. Microdissection after fixation is preferable, particularly for RNA analysis.

Materials

Low-cellularity sample: fine-needle aspiration or cultured cells suspended in medium
95% and 70% ethanol
Cytospin instrument and appropriate single sample chamber cytospin device (e.g., Shandon/Lipshaw)
Glass slides, clean

Assemble and load cytospin devices

1. Assemble the sample chamber cytospin device with clean glass slides according to the manufacturer's instructions.

Plain uncoated, charged, or silanized glass slides can be used.

2. Load the assembled collection chamber devices into the support plate of the cytospin instrument.

   They must be secure, freely tiltable, and symmetrically distributed.

Add samples and spin

3. Pipet low-cellularity sample into sample chambers.

   The optimal amount of specimen will vary with its cellularity. Samples of low cellularity will require 300 to 400 μl per chamber; highly cellular samples will require only 100 to 200 μl per chamber.

4. Press closure cap on each sample chamber.
5. Lock the lid of sealed head and close the cytospin cover.
6. Program cytospin for 3 min at 1500 rpm on high acceleration and press start.

Rapidly fix cytospins

7. When the alarm signaling the end of the spin sounds, quickly remove the assembled collection chambers. Open the chambers and remove the slides by lifting the blotter away from the slide

   This method avoids damage of cell membranes and thus smearing.

8. Quickly transfer slide into 95% ethanol without allowing the specimen to dry. Fix 10 min. Transfer slide to 70% ethanol for 30 sec.
9. Proceed to H&E staining (see Basic Protocol 5) or other stain of choice.

REMOVING BLOOD FROM SAMPLES FOR CYTOLOGIC SMEARS OR CYTOSPINS

ALTERNATE PROTOCOL 1

Particularly bloody specimens may benefit from separating red blood cells from other cellular elements, thereby concentrating the desired cells (especially epithelial cells). This can be accomplished by utilizing the Ficoll-Paque density gradient technique described here. The specimen is layered onto an undiluted Ficoll-Paque solution and centrifuged. Differential migration during centrifugation results in the formation of layers enriched in different cell types. This allows extraction of other cells in the sample from red blood cells. This method is not ideal for isolating white blood cells for microdissection as many of them separate with the red blood cells. See the Arcturus Engineering web site (*http://www.arctur.com*) for a protocol for isolating the buffy coat of blood.

Materials

Cytologic sample
Sterile saline (i.e., 0.9% w/v NaCl) or balanced salt solution
Ficoll-Paque (Pharmacia)
50-ml centrifuge tubes

Concentrate cellular components

1. Centrifuge the cytologic sample for 10 min at 350 × *g*, room temperature, in a 50-ml centrifuge tube.
2. Aspirate the supernatant with a pipet.
3. Resuspend the cell "button" in 5 to 10 ml sterile saline or balanced salt solution.

Separate cellular components

4. Add 20 ml Ficoll-Paque to a clean-50 ml centrifuge tube. Carefully pipet the cell suspension onto the Ficoll-Paque.

   It is best not to mix the Ficoll-Paque with the specimen at this point.

5. Centrifuge 10 min at 350 × *g*, room temperature.

   After centrifugation, the top and clearest layer contains any epithelial cells and some white blood cells. The middle layer is the Ficoll-Paque and the lowest layer is predominantly red blood cells and white blood cells.

6. Prepare the superficial cell layer as direct smears or cytospins (see Basic Protocols 3 and 4).

BASIC PROTOCOL 5

HEMATOXYLIN AND EOSIN STAINING

Histologic section and cytologic preparations must be stained so that the component cells can be adequately visualized for accurate identification; hematoxylin and eosin stain is commonly used for this purpose. With this stain, nuclei are black-blue and cell cytoplasm and most extracellular material are varying shades of pink. Although both hematoxylin and eosin staining solutions can be prepared from their basic components, the authors recommend purchasing prepared, ready-to-use stains.

Materials

Sample on a glass slide (see Basic Protocols 1 to 4)
70%, 95%, and 100% ethanol
Sterile, distilled, or RNase free water
Mayer's hematoxylin (Richard-Allan Scientific)
Bluing reagent (Richard-Allan Scientific)
Eosin Y
Xylene

1. *For frozen sections (optional):* Rapidly remove the sample on a glass slide from −80°C storage (see Basic Protocol 1) and immerse in or flood with 70% ethanol without allowing the slide to thaw and dry prior to contact with the ethanol. Allow the ethanol to remain in contact with the tissue for 30 sec.

   Deparaffinized fixed sections (see Basic Protocol 2) as well as samples prepared by direct smear or cytospin (see Basic Protocols 3 and 4) will already be in 70% alcohol and are ready to proceed through the following steps.

2. Allow the slide containing the tissue section to remain in the following solutions for the specified times in the specified sequence:

Sterile, distilled, or RNase-free water	10 sec
Mayer's hematoxylin	10 sec
Sterile, distilled, or RNase-free water	10 sec
Bluing reagent	15 to 30 sec
70% ethanol	15 to 30 sec
Eosin Y	15 to 30 sec
95% ethanol	30 sec
95% ethanol	30 sec
100% ethanol	30 sec
100% ethanol	30 sec to 1 min
Xylene	1 to 5 min

3. Allow the section to air dry completely and proceed to LCM (see Basic Protocol 6 and Alternate Protocol 2).

   Poor LCM transfers will result if the tissue section is not fully dehydrated. This may result if the 100% ethanol becomes hydrated after repeated use. One way to check the 100% ethanol for water is to put a small amount into xylene. If there is water present, the xylene will become cloudy. The final xylene rinse also facilitates the efficiency of transfer with LCM. If a tissue section does not transfer well, repeating the dehydration with fresh 100% alcohol and/or a longer xylene rinse may help. While other staining protocols can be used, the slides should be dehydrated with graded alcohols and the final xylene step.

LASER CAPTURE MICRODISSECTION

BASIC PROTOCOL 6

The described procedure is for the PixCell I or II Laser Capture Microdissection System and assumes a general knowledge of the function of the components of the instrument and the software that accompanies the instrument. The general theory underlying the use of the instrument is discussed elsewhere (see Background Information). The procedure can be divided into three basic steps: slide positioning, microdissecting with the laser, and collecting the microdissected cells. Additional information about the Arcturus LCM software, including capturing and storing images, and additional instruction for LCM, can be found in the instrument users' manual and at the Arcturus Engineering web site (*http://www.arctur.com*), the National Institute of Environmental Health Sciences web site (*http://dir.niehs.nih.gov*), or from Arcturus technical support (650-962-3020).

Materials

Glass slide with stained specimen (see Basic Protocol 5)
Appropriate lysis buffer (e.g., DNA lysis buffer, protein lysis buffer; see recipes)

PixCell I or II Laser Capture Microdissection System (Arcturus Engineering)
Arcturus LCM software (Arcturus Engineering; optional)
CapSure transfer film (Arcturus Engineering)
0.5-ml microcentrifuge tubes (Eppendorf)

NOTE: Wear gloves when microdissecting to avoid contamination of the LCM specimens. Clean the microscope stage and capping station before beginning the microdissection (e.g., use 95% ethanol wipes), to reduce the possibility of contamination.

Position slide (section) to be microdissected

1. Turn on the PixCell I or II Laser Capture Microdissection System. Open the Arcturus LCM software if it is to be used.

   The Arcturus LCM software is not required for LCM as all adjustments of parameters can be made on the laser electronics box; however, it eases the use of the instrument and performs useful functions, such as counting the pulses of the laser ("shots") and allowing the procurement and archiving of images.

2. Place the glass slide with the stained section to be microdissected on the microscope stage. Move the joystick so that it is perpendicular to the tabletop to allow proper placement of the CapSure transfer film ("cap"). Focus the microscope to view the tissue or cells. Locate the area to be microdissected, moving the slide by hand rather than with the joystick, so that the joystick will be in proper alignment when the area to be microdissected is located.

   Samples are usually stained in order to be visualized for LCM; however, LCM can be performed successfully without staining, but desired cells may not be identifiable.

   The area selected should be located such that a portion of the slide covers the vacuum chuck hole and the slide spans the central hole in the stage.

3. Turn on the vacuum slide holder.

   IMPORTANT NOTE: *The joystick should now be used to move the slide.*

4. Use the visualizer to more precisely locate the cells to be microdissected.

   The light from the microscope will need to be increased when using the visualizer.

   The area to be microdissected should be in the field of view.

   The sections are not coverslipped; therefore, the area of interest may be difficult to visualize. All models of the PixCell System are equipped with a visualizer which acts to diffuse light and improves resolution; however, the visualizer is engaged differently on different models (see instrument user's guide).

Microdissect with the laser

5. Pick up a cap from the loaded cassette module on the right side of the microscope stage (see instrument users' guide for instructions on loading the caps into the cassette module) with the placement arm. Swing the placement arm toward the caps until the arm overrides the first cap in the cassette module. Ensure that the cassette module is engaged in the proper indent so that the first available cap is aligned with the arrow on the microscope stage. Lift the transport arm until the cap detaches from the base slide in the cassette module.

6. Without lowering the placement arm, swing the arm back toward the tissue section as far as possible, so that the arm is over the tissue. Make sure that the area to be microdissected is still in the microscopic field of view by looking through the microscope eyepieces or at the monitor. Gently lower the arm so that the cap contacts the tissue section.

   If there are folds in the tissue, the cap may not make direct contact with the entire surface in the area to be microdissected, and transfer efficiency will be compromised; therefore, it is advisable to inspect the tissue before placing down the cap. If any tissue is mounded or folded, it is best not to place the cap over that area. Alternatively, the area of the tissue with folds can be scrapped off the slide using a sterile razor blade, leaving only flat portions of the tissue section. The tissue section must be dry and cannot be coverslipped for LCM transfer.

7. Enable the laser by turning the key on the laser electronics box and pushing the laser-enable button.

   The laser-tracking beam should now be visible on the monitor, as well as the area to be microdissected. If it is not, try lowering the light from the microscope or raising the intensity of the tracking beam. If it is still not visible, check that the laser is enabled and that the joystick is perpendicular.

   Avoid passing hands through the path of the laser when it is enabled.

8. Using the 20× objective, adjust the focus of the tissue by moving the slide via the joystick to an area of the slide without tissue. Adjust the laser spot size to 7.5 µm. Lower the light from the microscope until there is a black monitor screen, except for the tracking beam. Turn the laser focusing wheel until the tracking beam is a bright spot with a well-defined edge.

   There should be no bright rings surrounding the central spot (Fig. 25A.1.1).

   Always focus the laser with the 7.5-µm spot. Each tissue section and slide will need to be refocused. Once the 7.5-µm spot is focused for a particular slide, there is no need to refocus the 15-µm or 30-µm spots, as they are automatically calibrated.

9. Adjust the laser power and pulse duration settings for the particular spot size to be used as provided below:

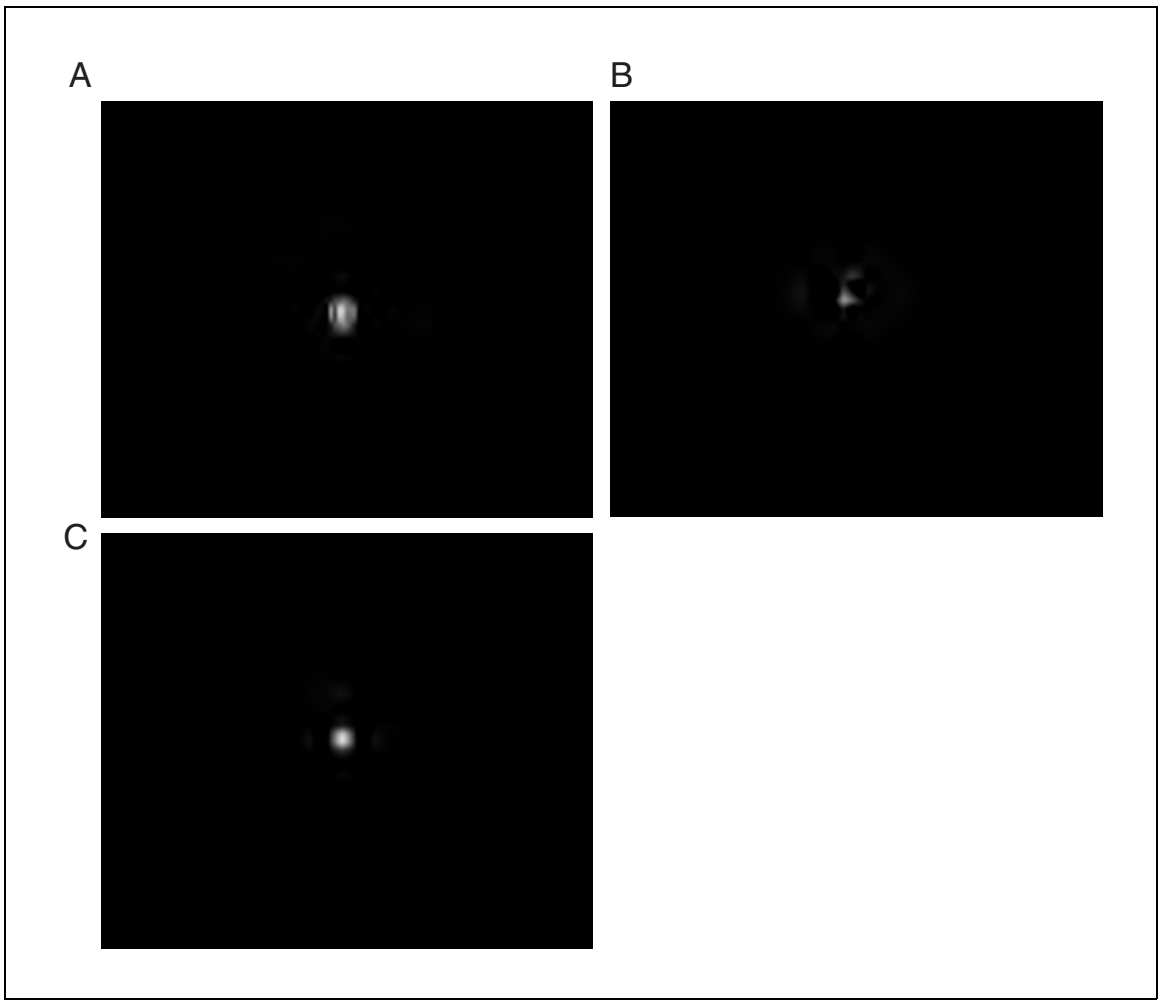

Figure 25A.1.1 Focusing the Laser Beam. (**A**) Unfocused beam, the spot of light has concentric halos of light. (**B**) Focused beam, the spot of light has a sharp border without halos of light. (**C**) Unfocused beam, the spot of light has a blurred border. A 20× objective and 7.5-µm spot size is used in all three pictures.

Spot size	Power	Duration
7.5 µm	40 mW	450 µsec
15 µm	25 mW	1.5 msec
30 µm	20 mW	5 msec

Laser power and duration determine the spot size. The power and duration settings given above should provide a melted area that is similar in size to the tracking beam at each of the three settings, but may require adjustment. See the user's manual for more information.

10. While the tracking beam is still located in an area without tissue, fire the laser by clicking the red button on the remote thumb switch to assess the effectiveness of the laser focus and settings.

    Effective melting ("wetting") of the polymer on the lower surface of the cap is indicated by a circle with a well defined black outline (see Fig. 25A.1.2).

    If the edges of the circle are not well delineated, check to make sure that the tissue section where the cap is placed is flat and refocus the beam. If this fails, increase the power and/or duration gradually and as little as possible (see Troubleshooting).

11. Test the effectiveness of LCM in the tissue section by moving the tracking beam to the cells to be microdissected. After targeting the cells, fire the laser. Move the slide

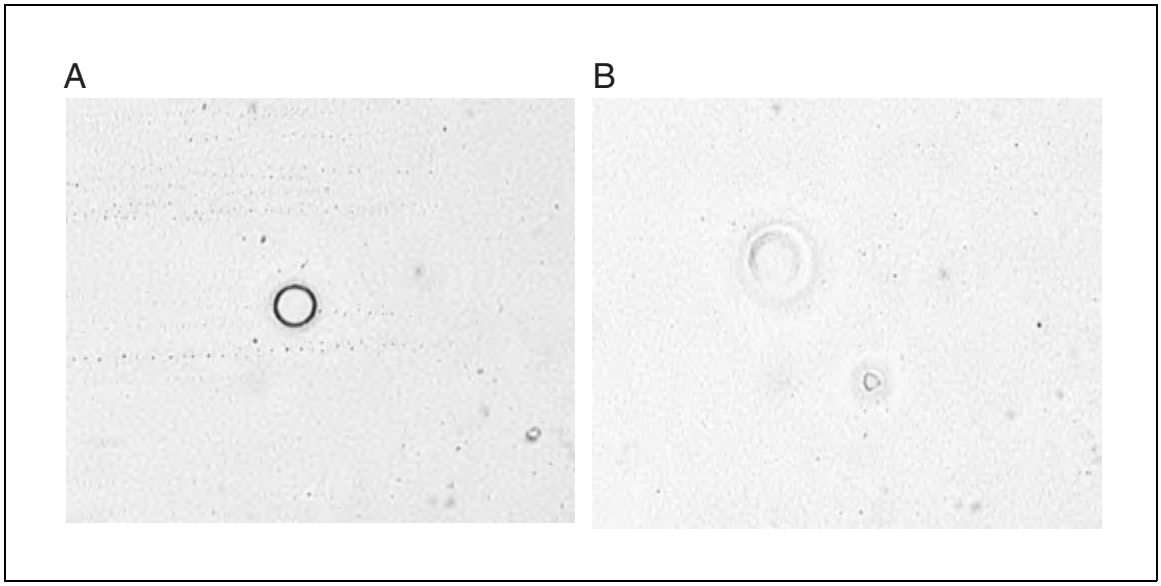

Figure 25A.1.2 Polymer Melting After Laser Firing. (**A**) An adequate and effective melt has a sharp, delineated border. (**B**) The border of an inadequate melt is blurred and indistinct.

with the joystick to another group of cells and fire the laser again. Limit the number of pulses for this test to two or three.

The delineation of the circle may be more difficult to visualize on the tissue section, but the tissue in an area of proper "wetting" should become more sharply focused because the melted polymer acts as a coverslip. Lift the placement arm and inspect the area in which the laser was fired for removal of cells (see before and after photomicrographs in Fig. 25A.1.3).

If the LCM was successful, the area where the polymer was melted should no longer be occupied by tissue and should be empty, although a small amount of cellular and stromal material may remain. The great majority of the tissue that occupied those spots should now be attached to the cap. This can be checked by releasing the vacuum slide holder, moving the slide so that a clean area without tissue is in the microscopic field of view, lowering the cap to the slide, and scanning the surface of the cap. The microdissected tissue should be visible on the cap surface. If this is not the case, there are several explanations and potential remedies (see Troubleshooting).

Avoid lifting and lowering the cap repeatedly after firing the laser and capturing some tissue. It is difficult to replace the captured tissue in the exact spot from which it came. Consequently the captured tissue, and tissue that may nonspecifically stick to the cap, will be placed on the histologic section, resulting in a layering effect which can limit contact of the cap with the tissue and compromise the effectiveness of LCM; therefore, limit the number of shots used to test the adequacy of capture, and, if the test capture is successful, avoid lifting the cap again until the microdissection is complete.

Dense, dark or thick samples may occlude the tracking beam. If this occurs, increase the intensity of the tracking beam.

12. Once LCM is achieved successfully with the test pulses, proceed to microdissect the remainder of the desired cells.

Collect microdissected cells

13. After completing the intended microdissection, lift the placement arm. Assess the completeness of the capture by inspecting the microdissected tissue and the cap as described above.

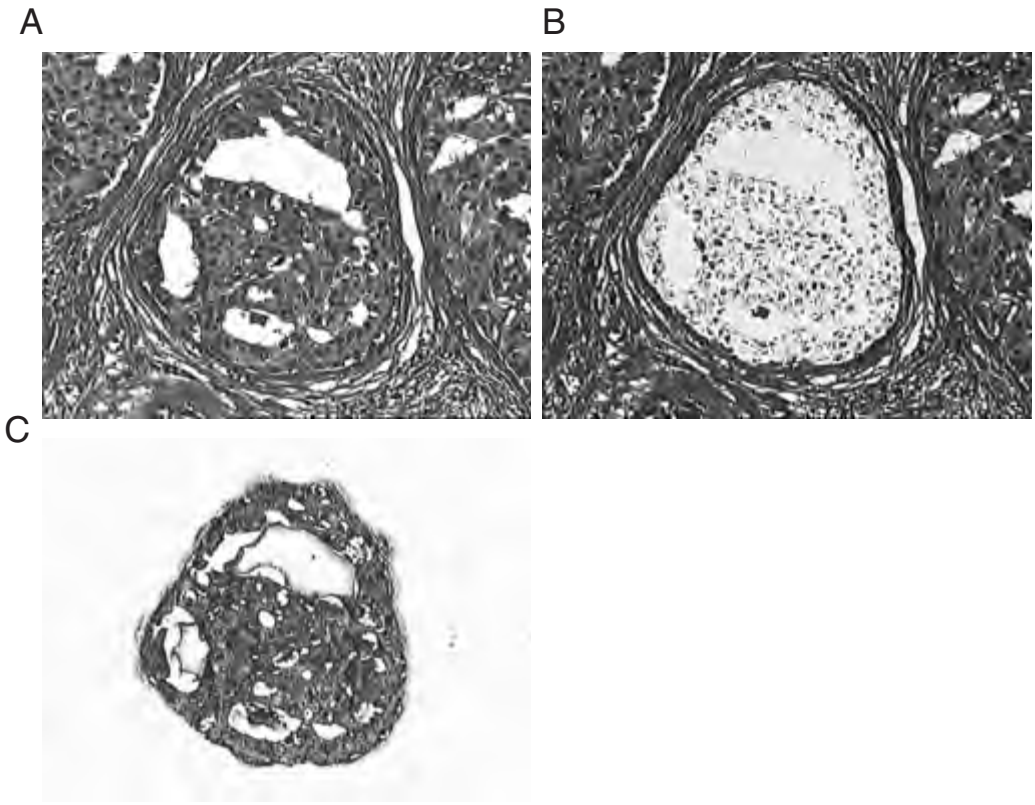

Figure 25A.1.3 LCM of ductal carcinoma in situ. (**A**) The area of ductal carcinoma in situ prior to LCM. (**B**) The same focus after LCM. (**C**) The microdissected focus on the transfer film (cap).

14. Swing the placement arm with the cap towards the right to the unload platform and place the cap in the designated slot. Move the placement arm, without lifting it, to the left and place in a resting position.

15. Using the cap insertion tool, pick up the cap from the unload platform by sliding the insertion tool along the guide rail until the cap is engaged in the tool. Remove the cap from the unload platform by lifting the insertion tool.

    The open end of the insertion tool should face the cap.

    Because tissue and cells that were not selected for capture may nonspecifically stick to the surface of the cap, it is important to remove this unwanted tissue. This can be accomplished by using the CapSure Pads (Arcturus Engineering), which have a sticky surface. If using the CapSure Pad, place the pad on the microscope stage in the path of the placement arm prior to placing the cap on the unload platform. Move the placement arm over the pad, lower the cap, and raise the pad to contact the cap. Raise the placement arm and the cap while holding the pad in place with your hand. A less costly alternative to the CapSure Pad is to use the sticky surface of Post-It Notes (3M). The Post-It Notes can be used after the cap has been removed from the unload platform. Peel a fresh Post-It Note off the pad and lower the cap, loaded into the insertion tool, to contact the sticky surface of the Post-It Note. Repeat this 2 to 3 times.

16. Using the insertion tool, insert the cap into a 0.5-ml microcentrifuge tube containing an appropriate amount of lysis buffer (e.g., DNA or protein lysis buffer), usually between 50 and 100 µl. Press down firmly and rotate the insertion tool to ensure an even seal.

The choice of lysis or digestion buffers is dependent on the analyte and the method of analysis. The recipes supplied in this unit (see Reagents and Solutions) provide examples of lysis buffers for DNA and protein that can be used for LCM samples. Other buffer recipes can be found in many of the references provided and at the BioProtocol web site (http://www.bioprotocol.com); however, it is best to customize the buffer to the methodology of the specific laboratory. The authors prefer to use Trizol (Life Technologies) or Stat-60 (Tel-Test) for cell lysis and RNA stabilization prior to RNA extraction and have not provided a recipe for an RNA lysis buffer; however, other buffers containing guanidine thiocyanate and 2-mercaptoethanol can also be used.

The caps fit well in standard 0.5-ml microcentrifuge tubes. When properly seated, the cap does not sit down fully in the tube, but should be seated evenly. Capped tubes will leak if the cap is pushed all the way down into the tube so that the top portion of the cap touches the lip of the microcentrifuge tube.

17. Invert the tube so that the lysis buffer contacts the cap surface. Flick the tube to move the lysis buffer to the cap surface, if necessary.

Place on ice or refrigerate until the microdissection session is over, if this will help to preserve the analyte in the chosen lysis buffer. This sample is now ready to be processed by appropriate methods for the analyte of interest.

ALTERNATE PROTOCOL 2

LASER CAPTURE MICRODISSECTION OF SINGLE OR A SMALL NUMBER OF CELLS

Arcturus Engineering has developed a line of related consumables that are specially designed for high-sensitivity capture and extraction of a single cell or a minimal number of cells. There are three key components of the system: a preparation strip that flattens the tissue section and removes loose debris, the high-sensitivity transfer cap (HS cap) that keeps the tissue surface area adjacent to the cells being captured out of contact with the sample, and a low-volume reaction chamber that fits onto the high-sensitivity transfer caps and accepts a low volume of lysis or digestion buffer while sealing out any nonselected material from the captured cells. The HS cap has a raised ridge on the contact surface so that only the ridge actually touches the tissue section. The surface coated with polymer only contacts the tissue in the area in which the laser is fired; thus, contamination by unwanted tissue is greatly reduced.

The basic steps of LCM as described (see Basic Protocol 6) are applicable to the use of the high-sensitivity consumables, with a few modifications. The modifications to the standard LCM protocol are described briefly below. These products can be purchased as a kit from Arcturus Engineering, which includes detailed instructions on their use.

Additional Materials *(also see Basic Protocol 6)*

Preparation strips (Prep Strips; Arcturus Engineering)
High-sensitivity transfer film (HS CapSure; Arcturus Engineering)
Tweezers, clean
Alignment tray designed for use with the high-sensitivity system
Low-volume reaction chamber (ExtracSure; Arcturus Engineering)

NOTE: All pipetting steps should be performed using filtered aerosol-resistant pipet tips.

Position slide

1. Prior to placing the stained sample on the glass slide on the microscope stage, apply a preparation strip (Prep Strip) to the tissue section or sample to flatten the tissue and remove loose debris.

2. Position the slide as described in the basic LCM protocol (see Basic Protocol 6, steps 1 to 4).

Microdissect

3. Pick up a high-sensitivity transfer film (HS cap; e.g., HS CapSure) from the loaded cassette module on the right side of the microscope stage (see instrument user's guide for instructions on loading the caps into the cassette module) with the placement arm and position the HS cap on the tissue to be microdissected. Enable and focus the laser as previously described (see Basic Protocol 6, steps 5 to 8).
4. Begin at a starting power of 75 mW and a pulse duration of 1 msec and make adjustments to the spot size by changing the duration setting rather than the power.

   These settings are those recommended for high-sensitivity LCM.

   For the smallest spot size, keep the duration and power settings low but pulse multiple times at the same target to ensure capture and transfer.

   The laser activates the transfer film, which then expands down into contact with the tissue. It is preferable to capture cells as close to the center of the cap as possible. Unlike basic LCM using the standard caps, the HS caps can be repositioned as often as needed to keep the targets toward the center of the cap, because the cap surface does not contact the tissue except at the area that the laser is fired. It is important to stay within the capture ring because areas outside the ring will be excluded from the low volume reaction tube.
5. Test the effectiveness as described (Basic Protocol 6, step 11).

Collect microdissected cells

6. After completing the intended microdissection, place the HS cap on the unload platform and pick up the HS cap with the cap insertion tool.
7. Remove the HS cap from the insertion tool using clean tweezers and place the HS cap into the alignment tray so that the captured sample is facing up.
8. Using clean tweezers, position the specialized low-volume reaction chamber over the cap.

   The chamber has a port for insertion of the appropriate lysis buffer (e.g., DNA or protein lysis buffer), which should be facing up.
9. Push the chamber down onto the cap until it snaps into place.
10. Pipet 10 µl desired buffer into the fill port. Cover the port with a 0.5-ml microcentrifuge tube or thin-walled PCR tube and press down to fit securely.
11. Proceed to extraction and analysis of the desired analyte.

TISSUE FIXATION AND PARAFFIN-EMBEDDING

SUPPORT PROTOCOL

If the researcher can choose a fixative, one which is alcohol based (e.g., 70% ethanol) is preferable for nucleic acid and protein recovery, and provides adequate morphologic detail for most LCM uses; however, alcohol-based fixatives have been reputed to confer a shrinkage artifact in histologic sections that is undesirable to diagnostic pathologists, as it results in tissue that is difficult to section and, at low dilutions, is inadequate for long-term storage of tissues (Vardaxis et al., 1997). On the other hand, Bostwick et al. (1994) successfully utilized an alcohol-based fixative in their pathology laboratory for one year without reporting these difficulties. Fixed tissue is typically embedded in paraffin to stiffen it so that thin histologic sections can be cut. Most paraffin used in pathology laboratories melts at ~60°C, which may accelerate formaldehyde reactions and damage RNA, DNA, and proteins; therefore, waxes or paraffins that have a lower melting point can be used, but they make softer tissue blocks that are more difficult to cut and may

require refrigerated storage. Tissue processing, embedding, and sectioning are generally performed in a histology laboratory by histotechnologists and generally require some degree of training and skill. The processing steps provided are suggested for utilization by histology laboratories processing tissue for LCM (*http://www.arctur.com*); however, other processing sequences may also provide good LCM results.

Materials

Fresh tissue
Fixative of choice (e.g., 70% ethanol)
Neutral buffered formalin (NBF; Richard-Allan Scientific)
70%, 80%, 95% and 100% ethanol
Xylene
Embedding paraffin

Tissue cassettes
Automated tissue processor
Embedding mold (Tissue-Tek)
Embedding center (optional; Leica)

Fix tissue

1. Place fresh tissue in a volume of fixative that is ≥10× the tissue volume, so that the fixative surrounds the tissue on all sides.

   Unfixed tissue that floats should be covered by a layer of gauze or paper towel to ensure the tissue is under the fixative. Fixation can be carried out at room temperature or 4°C.

   Fixation at 4°C slows down the autolytic process and can be useful for larger specimens.

2. Fix the tissue for an appropriate amount of time.

   The time required for fixation is dependent on the size of the tissue and the speed with which the fixative penetrates the tissue. Formalin and 95% ethanol penetrate at a rate of ~1 mm/hr. Fixation time and tissue size should be adjusted as necessary.

   For any fixative used, a fixing period of 16 to 24 hr is recommended to provide complete tissue fixation; however, a fixation period of <6 hr provides better recovery of DNA than longer fixation times (http://www.arctur.com).

3. *Optional:* Trim tissue sections from larger fresh or fixed tissue specimens so that they are no more than 3 mm in thickness and no larger than the dimensions of the cassette used for tissue processing. Place one section in each cassette.

   Again, 1-cm maximum dimension is ideal.

Process and embed tissue

4. After the tissue sections in the cassettes are fixed, place the cassettes in the first station of an automated tissue processor. Program and load the processor.

5a. *For routine overnight processing:* Perform the steps in Table 25A.1.1.

   After processing, the tissue will be infiltrated with paraffin.

5b. *For accelerated processing:* Follow the steps in Table 25A.1.2.

   No difference has been found in the LCM transfer efficiency of tissues processed either way.

6. Remove the tissue from the original cassette and embed the paraffin-infiltrated tissue in additional melted paraffin in an embedding mold. Allow to cool and harden.

7. Adhere the paraffin block to a cutting platform (chuck) and remove the paraffin block from the embedding mold. The paraffin block is now ready for sectioning (see Basic Protocol 2).

   Also see Sheehan and Hrapchak (1987b).

Table 25A.1.1 Routine Overnight Tissue Processing

Station	Solution	Concentration	Time (min)	Temperature (°C)
1	NBF[a]	10%	2:00	40
2	Ethanol	70%	0:30	40
3	Ethanol	80%	0:30	40
4	Ethanol	95%	0:45	40
5	Ethanol	95%	0:45	40
6	Ethanol	100%	0:45	40
7	Ethanol	100%	0:45	40
8	Ethanol	100%	0:45	40
9	Xylene	100%	0:45	40
10	Xylene	100%	0:45	40
11	Embedding paraffin	—	0:30	58
12	Embedding paraffin	—	0:30	58
13	Embedding paraffin	—	0:30	58
14	Embedding paraffin	—	0:30	58

[a]If neutral buffered formalin (NBF) is not the initial fixative, skip station 1.

Table 25A.1.2 Accelerated Tissue Processing

Station	Solution	Concentration	Time (min)	Temperature (°C)
1	Ethanol	70%	0:10	40
2	Ethanol	80%	0:10	40
3	Ethanol	95%	0:15	40
4	Ethanol	100%	0:20	40
5	Ethanol	100%	0:30	40
6	Xylene	100%	0:30	40
7	Xylene	100%	0:30	40
8	Xylene	100%	0:30	40
11	Embedding paraffin	—	0:30	60
12	Embedding paraffin	—	0:20	60
13	Embedding paraffin	—	0:30	60
14	Embedding paraffin	—	0:20	60

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

DNA lysis buffer

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
0.2% (v/v) Tween 20
100 µg/ml proteinase K

The authors use this lysis buffer for samples intended for PCR. Arcturus Engineering offers DNA extraction kits that were developed specifically for LCM specimens. The proteinase K should be stored at −20°C in aliquots, while the Tris·Cl and Tween 20 can be stored at −4°C. Once the proteinase K is thawed and added, the buffer should be used immediately.

Protein lysis buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2*)
0.1% Triton X-100
1.5 mM EDTA
10% (v/v) glycerol
Store several months at –4°C

This lysis buffer has been found to be useful for analysis of membrane-bound proteins (Simone et al., 2000). For cytoplasmic proteins, "T-Per" tissue protein extraction liquid reagent (Pierce Chemical) has been recommended (Simone et al., 2000). It has also been suggested that the addition of protease inhibitors, such as 4-(2-aminoethyl)-benzenesulfonyl fluoride (Boehringer Mannheim) to the buffer increases the yield of protein (Banks et al., 1999; Ornstein et al., 2000a).

COMMENTARY

Background Information

Technologic advances in gene sequencing and amplification techniques are allowing the identification of alterations in genes, proteins, and biochemicals that can explain the etiology and pathogenesis of many disease processes; however, the efficacy of these technologies depends on the identity and the purity of the cells being analyzed. Physical homogenization of tissues results in a mixture of many cell types—i.e., some are normal or minimally altered components, while others may be significantly diseased. Alterations detected in such homogenates cannot be localized to a particular cell type. Multiple mechanical methods for separating cells of interest from tissues have been described, especially as related to histologic sections (Sirivatanauksorn et al., 1999), but their methodology is time-consuming, extremely labor-intensive, and often imprecise. Laser Capture Microdissection (LCM) is one of the new generation of microdissection techniques that is relatively quick and precise.

LCM was conceived and first developed as a prototype research tool at the National Institute of Child Health and Human Development (NICHD) and the National Cancer Institute (NCI) of the National Institutes of Health (NIH). Arcturus Engineering and the NIH, working through a Cooperative Research and Development Agreement, developed LCM into a commercial laboratory instrument that is now utilized in many research laboratories. Other efficient microdissection techniques, such as laser pressure catapulting, have also been described (Bohm et al., 1997; Sirivatanauksorn et al., 1999).

With LCM, cells of interest are dissected from tissue sections or cytologic samples after microscopic identification with the aid of an ethylene vinyl acetate transfer film containing a near-infrared absorbing dye. The transfer film coats a flat surface of an optically clear plastic cylinder, the "cap," with a diameter of 6 mm. The LCM system places the transfer film in contact with a histologic section and then directs an invisible infrared laser pulse onto the overlying polymer. The laser pulse is absorbed by and melts the transfer film causing it to flow around the targeted cells. The polymer rapidly cools and creates a bond between the transfer film and the targeted cells. The targeted cells can then be lifted from the section and utilized for RNA, DNA, or protein analysis (Fig. 25A.1.4). This targeting and capturing can be repeated many times on the same tissue section or cytologic sample. The temperature rise in the tissue created by the laser is limited to 90°C (Suarez-Quian et al., 1999) and is transient, lasting only a few milliseconds. Experimental results indicate that DNA, mRNA, and proteins are not degraded by the LCM process (Goldsworthy et al., 1999; Suarez-Quian et al., 1999).

Critical Parameters

LCM can be performed on solid tissues that have been either frozen or fixed under specified conditions, cytologic smears, or cytospin preparations derived from animals or patient samples. The choice of specimen type depends on the type of tissue or cytologic specimen that is available, the physiologic or pathologic condition to be investigated, and the molecule to be analyzed (i.e., DNA, RNA, or protein). Solid tissues are typically sectioned for histologic examination, whereas cells from blood or cytologic samples, such as fine-needle aspirates, are prepared as direct smears or cytospins. Frozen tissues have the benefit of being processed more rapidly for LCM than fixed tissue and are considered to be the most reliable source for

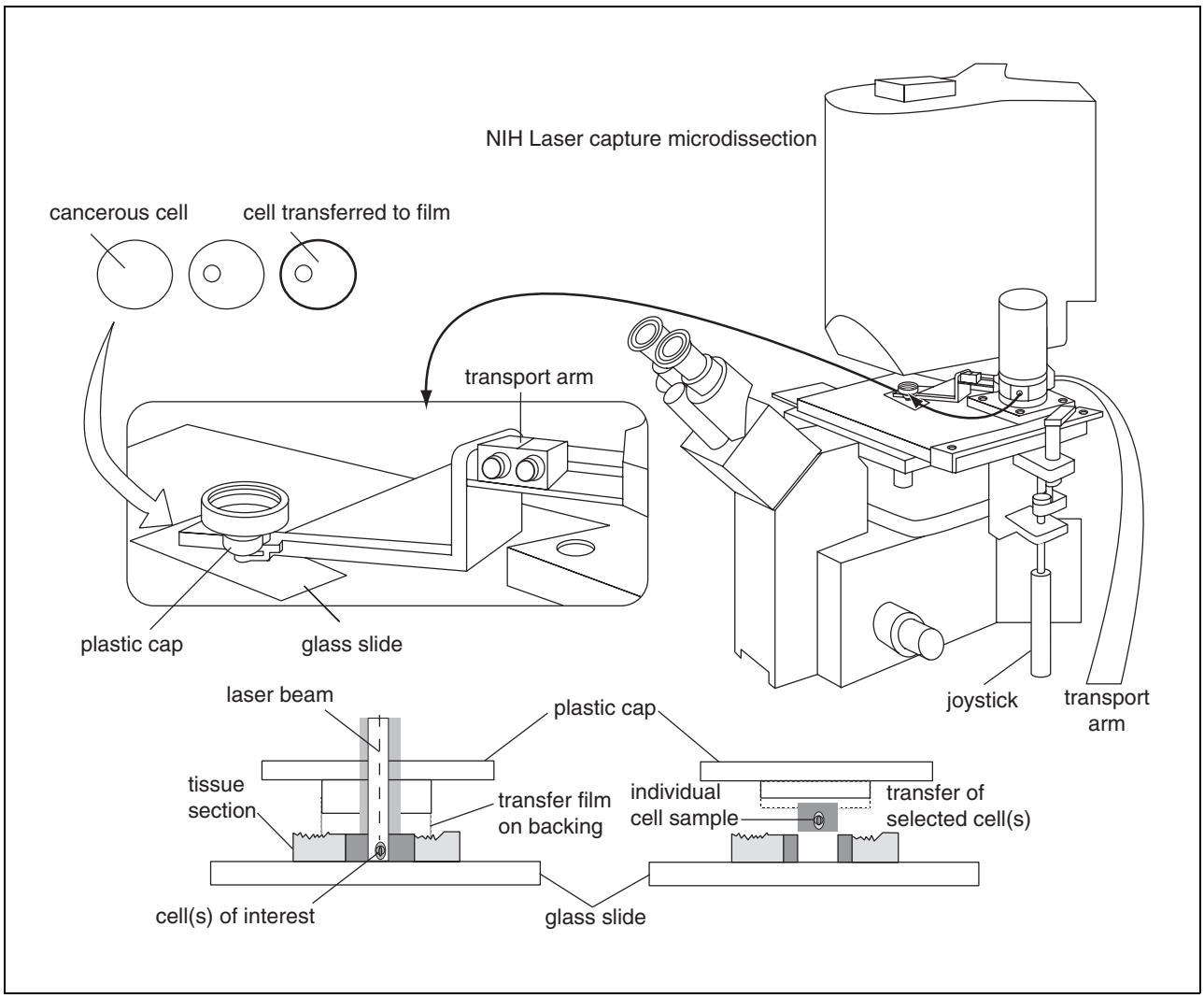

Figure 25A.1.4 LCM Instrument. Schematic of the operation of the PixCell Laser Capture Microdissection Instrument, indicating the location of the transfer arm, transfer film ("cap"), and the glass slide with the specimen to be microdissected. Also shown is a cross-section of the tissue specimen with overlying cap demonstrating the effect of laser firing. Reprinted with permission from Bonner et al. (1997).

molecular (i.e., DNA, RNA, and protein) recovery. Lengths of RNA and DNA of up to 800 base pairs have been recovered from sections prepared from frozen tissue (*http://www.arctur.com*; Dietmaier et al., 1999; Shibutani et al., 2000); however, histologic and cytologic detail are poor compared to fixed paraffin-embedded tissue and subtle diagnostic features may be difficult to discern. The most frequently utilized tissue fixative is neutral buffered formalin (NBF; i.e., 10% buffered formaldehyde) followed by paraffin embedding to allow histologic sectioning. This combination results in cross-linking and "breakage" of proteins, RNA, and DNA, which must be considered when utilizing tissues prepared in this manner.

Regardless of the preparation, cells or tissue are usually stained in order to be visualized for LCM, although LCM can be performed successfully without staining. Hematoxylin and eosin (H&E) stain is the most commonly used stain for examination of histologic sections, and diagnostic histopathologic criteria are based on its use in veterinary and human pathology practice; therefore, it is frequently used for LCM even though hematoxylin may bind to nucleic acids causing adverse effects during PCR. Other stains such as methyl green and nuclear fast red have been recommended as alternatives, and literally hundreds of others exist in clinical practice and for research applications (Ohyama et al., 2000); however, H&E stained LCM samples have recently been shown to amplify equally as well as samples stained with methyl green, toluidine blue O, or azure B (Ehrig et al., 2001). This is likely due to the relatively small size of LCM samples,

which thus contributes only a small amount of hematoxylin to the PCR reaction mix. It is also recommended that the duration of staining with hematoxylin be minimized to decrease the concentration present. Eosin has been reported to interfere with PCR analysis utilizing the TaqMan instrument and can appear on electrophoretic gels when relatively large numbers of cells are captured for protein analysis (Banks et al., 1999; Ehrig et al., 2001). Consideration should be given to minimizing or eliminating its use when samples will be utilized for either of these assays. Specimens can also be stained immunohistochemically or with fluorescent labels prior to microdissection (Fend et al., 1999b; Murakami et al., 2000).

There are two alternative methods for specimen staining. One is to place the staining solutions into either Coplin jars or staining dishes and immerse the slides in the appropriate solutions. If this method is used, the stains should be changed frequently to prevent contamination by tissue fragments from other tissue samples or microorganisms found in the environment, and to avoid excessive dilution of the staining solutions. The second alternative, and the one that the authors prefer, is to keep the solutions in clean plastic squirt bottles and use a slide staining rack. The slide to be stained can then be placed on the staining rack and the solutions can be applied gently to the slide to cover the tissue or cells, allowed to remain the appropriate time, and then drained from the slide and replaced by the next solution. This reduces any possible contamination, minimizes dilution of solutions, and has the added advantage of using less reagents. For solutions requiring a duration of contact with the slide that is longer than 1 min (i.e., xylene), we utilize small Coplin jars. Thus, the best features of both systems may be used efficiently.

For a successful LCM transfer, the polymer film must be bonded to the targeted tissue so it forms a stronger bond than that between the tissue and the underlying glass slide; therefore, proper sample preparation is critical. It is important that the sample be well dehydrated so that the melted polymer can infiltrate intercellular spaces and create a tight bond. The final dehydration and xylene steps have been found to be absolutely crucial for successful LCM. Any moisture present in the sample during LCM will give less than optimal results. Ideally, samples should be microdissected shortly after dehydration; however, samples can be stored with desiccant after staining and dehydrated for later microdissection, although this is not recommended for recovery of RNA because of its lability. Additionally, the humidity in the laboratory will also affect the results, and protocols may need to be modified accordingly. Other factors that will affect this bond are presented below (see Troubleshooting).

Specimens, reagents, and materials for processing must be handled in a manner that will allow optimal preservation of the molecule to be analyzed; therefore, samples for RNA and DNA should be handled to minimize contamination from other tissues. Samples for RNA analysis should be processed rapidly, either as fresh-frozen material or briefly fixed in 95% ethanol. RNase-free reagents and materials should be utilized whenever possible. Also, the duration of the actual microdissection session on each stained frozen section should be limited to less than 30 min for optimal RNA preservation. Samples for protein analysis are also best processed as for RNA analysis, but reagents that include protease inhibitors can be used. DNA is more stable, and fixed or frozen tissues can be used, but samples should not be overfixed in formalin, as DNA yield increases with prolonged fixation times (<6 hr is preferable for small samples).

Troubleshooting

If LCM fails to capture the cells (i.e., they are not released from the slide), the following steps are recommended.

1. Refocus the beam (see Basic Protocol 6).
2. Make sure the sections are flat. Wrinkles can be shaved off using sterile razor blades. Dip the section in xylene after saving the wrinkles to make sure that no contaminating debris remains on the section.
3. Change the cap. Not all caps perform equally well and the age of the caps is important. It is best not to use expired caps and to buy relatively small numbers of caps at a time so that the stock is relatively new.
4. Ensure thorough dehydration of the specimen. Place the slides in fresh xylene for 1 min or more and allow drying in a biologic safety hood for 1 to 5 min. If LCM is still not successful, pass the slides through 95% ethanol twice for 30 sec, absolute ethanol twice for 30 sec, and xylene for 1 to 5 min.
5. Process a new section and make sure that the frozen sections or cytologic specimens have not been allowed to dry on the slide prior to fixation. For formalin-fixed sections, do not bake or at least decrease the baking time.
6. Try a different brand or type of glass slide.

7. If still not successful, call the technical support at Acturus Engineering (650-962-3020). The authors also find that talking with other researchers working with LCM to be very useful.

If LCM is successful, but the cap contains contaminating debris, the following measures are recommended.

1. Make sure the slide is free of debris. It may be necessary to wash the slide in fresh changes of xylene.
2. Use a CapSure Pad or Post-It Note to remove any debris from the cap.
3. Use HS caps, which minimize contamination.

If the LCM was successful, but no RNA, DNA, or protein was identified at analysis, try the following.

1. Make sure optimum laboratory practices and conditions that are free of nucleases or proteinases have been observed.
2. Check the cap to see if the microdissected tissue has dissolved in the lysis buffer in the microcentrifuge tube.
3. Increase the number of microdissected cells.
4. An overnight incubation at 37°C can be used to lyse the cells from the cap when using DNA lysis solutions, if required. For RNA and proteins, inverting and gentle agitation should be used to dislodge the cells from the cap.

Anticipated Results

Many molecular analyses have been successfully performed on cells procured by LCM. These include genomic analyses such as loss of heterozygosity analysis, restriction fragment length polymorphism (RFLP) analysis, DNA methylation analysis, fluorescence in situ hybridization, and comparative genomic hybridization (Finkelstein et al., 1999; Guan et al., 1999; DiFrancesco et al., 2000; Jones et al., 2000; Shen et al., 2000; Slebos et al., 2000). Gene expression analysis has been accomplished from LCM samples utilizing reverse transcription PCR, construction of cDNA libraries, and differential hybridization on high-density-spotted nylon filters or glass microarrays (Peterson et al., 1998; Fend et al., 1999b; Kuecker et al., 1999; Luo et al., 1999; Sgroi et al., 1999; Garrett et al., 2000; Leethanakul et al., 2000; Ohyama et al., 2000). Successful proteomic analysis has been accomplished by coupling LCM with immunoblotting (*UNIT 10.8*), solid-phase sequential chemiluminescent immunometric assay, one-dimensional and two-dimensional polyacrylamide gel electrophoresis (PAGE; *UNITS 10.2-10.4*), protein chip surface enhanced laser desorption/ionization (SELDI) mass spectrometry, as well as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Wright et al., 1999; Natkunam et al., 2000; Ohyama et al., 2000; Ornstein et al., 2000a,b; Palmer-Toy et al., 2000; Simone et al., 2000; also see *UNIT 10.21*). For all these assays, the expected results will depend on the quality of preservation of the analyte of interest within the sample and upon procurement of at least the minimum number of cells required for analysis.

The number of cells captured depends on tissue thickness and type, the size of the cells, and the size of the laser spot. The number of cells procured can be estimated by counting the number of cells per spot and multiplying by the number of pulses of the laser. The transfer efficiency of the capture should also be considered and can be assessed by viewing the captured tissue on the cap and estimating the percentage of spots that contain tissue.

The number of cells required depends on the assay and whether formalin-fixed, alcohol-fixed, or frozen samples are used. A single PCR reaction (DNA analysis) can be successfully performed with a single cell; however, results are more reliable with at least 10 to 20 cells from a 10-µm-thick, formalin-fixed, paraffin-embedded section. Such small quantities of cells may not account for the significant heterogeneity that exists even within populations of the same cell type, which should be considered when determining the number of cells to be used. For RNA analysis, fresh-frozen tissues and cytologic specimens briefly fixed in alcohol are preferred. Only a small number of cells (i.e., <50) may be required for transcripts of high copy number per cell when utilizing RT-PCR; however, the authors prefer using ≥1000 cells for RT-PCR. cDNA arrays require significantly more RNA, but how much will depend on the type and size of array. It is estimated that a typical mammalian cell contains ~20 pg total RNA/cell; therefore, to achieve 5 µg RNA, the lower limit for some expression arrays, will require the microdissection of 2.5×10^{11} cells, a daunting task. Thus, some authors have advocated amplification of RNA or resultant cDNA prior to hybridization with these larger arrays, even though this may introduce some degree of amplification bias (Luo et al., 1999; Ohyama et al., 2000). For protein analysis, using 50,000 cells for two-dimensional PAGE analysis has been a successful starting point. For western blot analysis, the number of cells

required is at least 2000 to 3000 (*http://www.arctur.com*). Some molecular assays may require modification in order to accommodate the relatively small amount of cells obtained by LCM.

Time Considerations

The time required for LCM is highly variable and depends on the method of tissue processing and staining, the number of cells to be microdissected, and the location and number of the desired cells in each section. H&E staining (see Basic Protocol 5) requires only 10 to 15 min. Microdissecting ~5000 cells, roughly equal to 1000 shots using a 30-µm spot size, will require 15 to 30 min, provided all the cells required are present within a single tissue section or sample. If multiple sections or samples are required to procure an adequate number of cells, the time required for staining additional sections should be added. This also assumes that the samples are well prepared and microdissected efficiently, and that the cells of interest are easy to identify and locate. Some skill is also required in operating the joystick in combination with laser firing and in being able to identify the tissue and cell type of interest.

The time required for lysis of the cells from the cap depends on the buffer and the method of sample preparation. We have found frozen tissue will be completely removed from the cap by Stat-60 after ~5 min. Formalin-fixed paraffin-embedded tissue in buffers containing proteinase K requires significantly more time and may require an overnight incubation at 37°C.

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Key References

Sheehan and Hrapchak, 1987. See above.

This is a standard reference used by many histotechnologists for the basics of tissue processing, embedding, and sectioning.

Suarez-Quian et al., 1999. See above.

This references provides a good overview of the mechanics and principles of LCM.

Internet Resources

http://dir.niehs.nih.gov/dirlep/lcm/guidelines.html

This website is maintained by the Laboratory of Experimental Pathology of the National Institute of Environmental Health Sciences and is another valuable source of protocols and general information for LCM.

http://www.arctur.com

This is the website of Arcturus Engineering. It is a very useful source of all LCM-related information including protocols, references, and resources. Many of the protocols that we use, including those presented here, are modifications of protocols found at this website.

http://www.bioprotocol.com

This website contains protocols for the performance of LCM, the preparation of tissues for LCM and for processing of microdissected tissue for DNA, RNA and protein analysis.

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Preparation of Single Cells from Solid Tissues for Analysis by PCR

The ability to amplify a few copies of DNA or RNA to analyzable quantities makes it technically possible to obtain detailed information regarding the DNA content and/or transcriptional pattern of a single cell (Mullis and Falona, 1987). Although in many cases, analysis at the level of the whole tissue can provide the required information, there are circumstances that necessitate acquiring data on individual cells of a particular type. A preparation of total DNA and RNA isolated from a tissue gives quantitative data but only an average profile, masking differences among individual cells. In situ analysis provides qualitative information on localization of abundant nucleic acids in specific cells, but is generally not quantitative. Thus, it can be desirable to apply quantitative assays to individual cells.

The acquisition of individual cells from blood and loosely associated tissues such as spleen is straightforward, since these organs are essentially cell suspensions. Solid tissues, however, are almost universally composed of tightly linked cells of multiple types, organized in a highly structured and functionally interactive manner (Gilbert, 1994). It is reasonable to expect that disruption of this environmental context rapidly alters the physiology of the once-partnered cells. Even in the case of easily dissociated tissues, the impact of manipulating the living tissue on the process under study must be considered. In addition, some adult cell types, most notably neurons, can be recovered only at low efficiency, with the majority bursting during the process (Pretlow and Pretlow, 1982).

This unit details a protocol for the separation of solid tissues into single-cell suspensions for subsequent analysis of nucleic acids and protein. This protocol was developed using mice, with the major focus being the analysis of the interaction of herpes simplex virus (HSV) with the neurons of the trigeminal ganglia (Sawtell, 1997). The balance between fixation and dissociation should be determined for the particular tissue of interest. It has been determined, however, that the dissociation protocol is directly useful for several other mouse tissues including liver, heart, skeletal muscle, lung, pancreas, brain, intestine, and reproductive organs. Kidney yields a combination of single cells and multicellular tubular structures. The adaptation of the method to other laboratory animals has not been fully explored. Again, the appropriate balance between fixation and dissociation would need to be determined for other species of interest. Using the approach of adjusting the volume of the fixative perfused through the animal to achieve this balance, the author's laboratory has determined that the method is directly useful for guinea pigs.

Tissues are fixed in situ by perfusion (see Basic Protocol 1), terminating cell processes and thus changes that would accompany dissociating the living tissue; their numbers can then be quantitated (see Support Protocol). Once separated, individual cells or groups of a particular cell type can then be analyzed using PCR strategies (see Basic Protocols 2 and 3; Fig. 25A.2.1). An alternative to fixing by perfusion (see Alternate Protocol 1) and a modification of the standard Percoll gradient separation to prepare *lacZ* expressing cells (see Alternate Protocol 2) are also provided. The method has broad potential and is particularly potent when the cell type of interest represents a minor population relative to other cells types in the tissue. The procedure can also be adapted to allow quantification of the number of cells within a tissue containing specific nucleic acid sequences, for example, a particular viral DNA or RNA sequence.

Contributed by N.M. Sawtell

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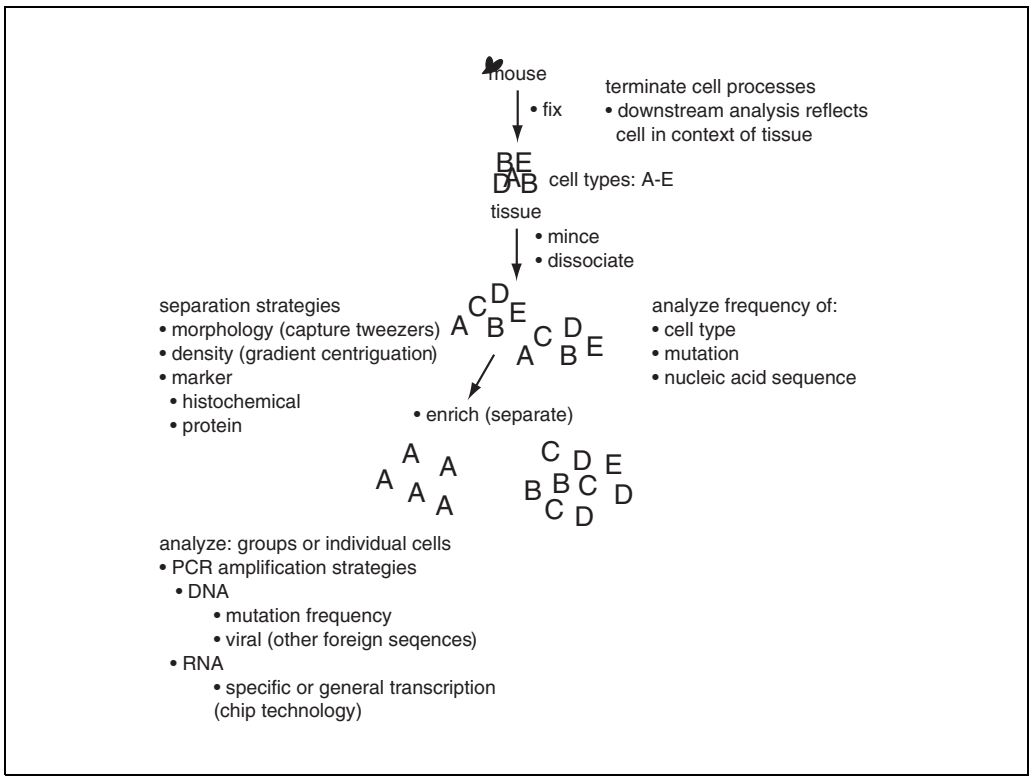

Figure 25A.2.1 Schematic representation of the preparation of single cells from solid tissue. Tissue is represented by the box in the center, and letters A to E represent different cell types within that tissue.

BASIC PROTOCOL 1

PERFUSION FIXATION AND ENRICHMENT OF SINGLE CELLS

In this protocol, the animal (here, a mouse) is perfused with Streck tissue fixative (STF), a noncrosslinking fixative. This fixative and the fixation conditions presented were determined empirically so that intracellular nucleic acids and proteins are preserved without interfering significantly with the ability of the dissociating enzymes to free the cells from the extracellular matrix. The fixed tissues of interest are dissected out, finely minced, and enzymatically separated using collagenase. The cell types of interest are then enriched using a suitable strategy.

At this point, any of a number of methods can be used to harvest the desired cell populations from the cell suspension. Percoll gradient separation is given here; however, the end application will strongly influence the procedure selected.

Materials

Streck tissue fixative (STF; Streck Laboratories)
Animal (e.g., mouse)
Sodium pentobarbital
95% ethanol
0.25% (w/v) collagenase CLS I (Worthington) in Hank's balanced salt solution (HBSS; see recipe)
Triple 0.2-µm filtered nanopure (3×F) H_2O
Percoll (Pharmacia): adjust to pH 6.0 with HCl

Peristaltic pump (BRL CP-600 or equivalent) and appropriate tubing
15- and 50-ml conical tubes
27-G needle
80°C water bath

Dissecting microscope (optional)
Clean dissection tools (e.g., forceps, scalpel blades, hemostat, 25-G needles)
Glass slides: bake overnight (3 hr minimum) at 250°C
200- and 1000-µl aerosol-resistant pipette tips
15-ml polystyrene conical tubes
9-in. Pasteur pipettes: bake overnight (3 hr minimum) at 250°C

Additional reagents and equipment for determining number of neurons recovered (see Support Protocol), and analyzing DNA or RNA from single-cell populations (see Basic Protocols 2 and 3)

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Depending upon the final application of the cells, all materials must be DNase- and RNase-free, and free of contaminating nucleic acids which could interfere with the interpretation of downstream PCR.

Perform perfusion fixation (as performed in mice)

1. Set up perfusion equipment by placing tubing from a peristaltic pump in the bottom of a ~50 ml conical tube containing 30- to 40-ml Streck tissue fixative (STF). Attach a 27-G needle to the other end (this will be inserted into the left ventricle). Run fixative through the line.

2. Place a 50-ml conical tube containing 50 ml STF in an 80°C water bath and equilibrate to temperature.

   Heat facilitates the inactivation of nucleases.

3. While the fixative is heating, anesthetize the animal by intraperitoneal injection of 80 to 100 mg/kg sodium pentobarbital. As soon as deep reflexes are fully deadened—i.e., in mice, lack of corneal reflexes (i.e., no blinking response when touched with the tip of a gloved finger) and response to pinching rear paw very firmly—place the animal ventral surface up on absorbent paper and wet the chest and abdomen with 95% ethanol.

   Isoflurane can be used as an alternative anesthetic.

4. Use forceps to lift the skin and, using a scalpel, make a T-shaped incision starting over the abdomen with the vertical- and horizontal-cut centers at the base of the sternum just below the diaphragm. Cut the diaphragm along the rib line and keep the chest cavity open by clamping the base of sternum with a small hemostat, rotating it upward toward the chest.

   For additional information on animal handling, see Coligan et al. (2001), Chapter 1.

5. Insert the needle at the end of the pump tubing into the left ventricle and start the pump, adjusting the flow rate to ~6 ml/min. When the right atrium becomes dilated, pierce with sharp pointed forceps to provide outflow. First pump 15 to 20 ml room-temperature STF through the animal to remove blood from the vasculature, followed by 40 to 50 ml heated (80°C) fixative. Stop the pump when fixative has been depleted.

   This procedure is not difficult but requires practice. The best indicator of a successful perfusion is paling of the liver. If the liver does not begin to pale rapidly, try repositioning the needle in the ventricle, adjusting its depth and angle.

Perfusion fixation is effective because the fixative is distributed to tissues and cells via the macro- and microvasculature. Coagulation of the blood in the vessels could occur upon contact with the heated fixative, thus it is important to first remove blood with room-temperature STF.

Dissociate tissue

6. Using a dissecting microscope (if possible), dissect the tissues of interest with "clean" dissection tools and finely mince on a nuclease-free (i.e., baked) glass slide using scalpel blades or needles (e.g., 25-G needles for ganglia).

   The author uses disposable instruments (e.g., unused 25-G needles, unused scalpel blades) that are discarded after use (i.e., a single dissection), which prevents the possibility of any carryover; however, it should be adequate to clean instruments in detergent (e.g., liquinox), rinse, and soak in 3% hydrogen peroxide for 2 hr, then rinse in 3×F H_2O and bake overnight (3 hr minimum) at 250°C. Any procedure for cleaning potentially contaminated instruments should be confirmed to be effective.

   Visualization of the mincing procedure under a dissecting microscope is helpful. Separate instruments must be used for each tissue unit if cross contamination will present a problem in the interpretation of downstream analyses.

7. Place minced tissue into 0.25% (w/v) collagenase CLS I in HBSS and incubate in a 1.5- or 2-ml microcentrifuge tube 5 to 10 min at 37°C.

   The volume of collagenase used will depend upon the amount of tissue. Six fixed mouse trigeminal ganglia (TG) are routinely digested in 1.5 ml collagenase.

   The investigator must screen batches of collagenase and select a batch that is free of DNase activity. If RNA will be analyzed, a batch free of RNase must be selected (see Critical Parameters and Troubleshooting, Collagenase)

8. After collagenase treatment, facilitate dissociation by gentle trituration, first using 1000-µl, then 200-µl (as the tissue dissociates into smaller pieces) aerosol-resistant pipet tips.

   In the author's studies, dissociation of TG is generally complete within 30 min.

   Depending on the application, the requirement for complete dissociation may be less critical. It is helpful to monitor progress of dissociation by viewing a drop of the suspension under the microscope.

9. Pellet dissociated tissue by microcentrifuging 5 min at 5000 rpm, room temperature. Resuspend gently in STF at room temperature. Heat resuspended cell suspension to 70°C for 10 min. Place on ice briefly, repellet, and resuspend in triple 0.2-µm filtered nanopure (3×F) water.

   At this point the integrity of the DNA, RNA, and/or protein (depending upon what will be analyzed), should be tested. DNA can be isolated using standard proteinase K/SDS digestion followed by phenol/chloroform extractions and ethanol precipitation (UNIT 2.1A). RNA can be isolated from the cells using commercially available reagents such as Ultraspec (Biotecx). When isolating RNA, cells should be homogenized using a tissue grinder to ensure complete disruption of the cell membrane. Protein should be prepared from cells by boiling in standard Laemmli cocktail (e.g., 0.125 M Tris·Cl/4% SDS/20% glycerol/10% 2-mercaptoethanol). Integrity of nucleic acid or protein is then determined by appropriate gel electrophoresis (Chapter 10). If information about the integrity of a specific nucleic acid or protein is desired, Southern (UNIT 2.9A), northern (UNIT 4.9), and/or immunoblotting (UNIT 10.8) can then be performed, probing the membrane with the relevant labeled nucleic acid probe or antibody. One should not necessarily expect that the integrity of these cells will be as great as that from tissue culture cells or fresh tissue, but it can be more than adequate to permit qualitative analysis by RT-PCR.

   Figure 25A.2.2A.1 to D.1 shows several tissues, including cerebral cortex, trigeminal ganglia, liver, and diaphragm, after fixation and dissociation.

10. Determine the number of neurons recovered (see Support Protocol).

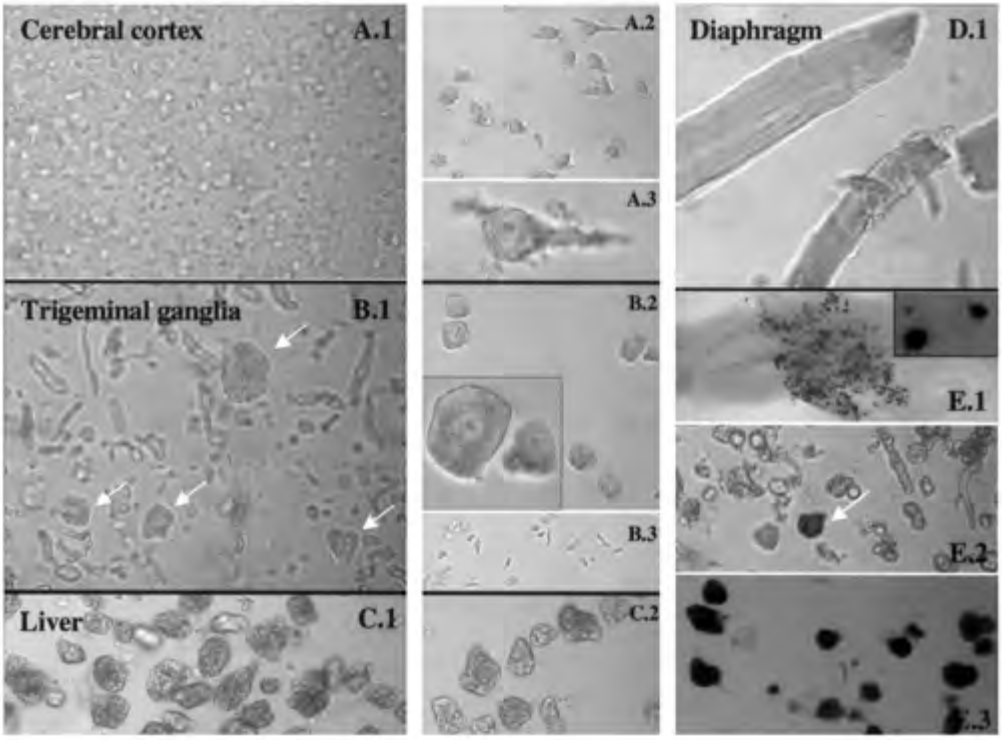

Figure 25A.2.2 Photomicrograph showing tissues after dissociation and density gradient centrifugation. Following perfusion fixation, several tissue types were removed, finely minced, and dissociated as described in this protocol. The total dissociated cell suspensions obtained from cerebral cortex (**A.1**), trigeminal ganglia (**B.1**), liver (**C.1**), and diaphragm (**D.1**) are shown. Following density gradient centrifugation, enriched populations of neurons were obtained from cerebral cortex (**A.2**, **A.3**) and trigeminal ganglia (**B.2**). Enriched populations of satellite and support cells isolated from trigeminal ganglia are shown in **B.3**. An example of "marker" based separation is shown in panels **E.1** to **E.3**. A mouse infected with a virus containing a β-galactosidase expression cassette was perfusion fixed and the trigeminal ganglia removed and stained histochemically for β-galactosidase activity using Xgal. The dark spots in panel E.1 are blue neurons, a result of the action of β-galactosidase on Xgal. The presence of this blue reaction product indicates that these neurons contain virus actively transcribing the β-galactosidase gene. The ganglia were then dissociated into single cell suspensions (E.2) and the blue neurons enriched by density gradient centrifugation (E.3). These neurons can be analyzed individually or in groups using PCR strategies.

11. Harvest desired cell populations by Percoll gradient centrifugation (steps 12 to 16) or by another suitable method.

    The end application will strongly influence the procedure selected. The following steps enrich for neurons, but the protocol can also be used to enrich for other cell types.

Enrich for neurons by Percoll gradient

12. Prepare a discontinuous Percoll gradient as follows:

    a. Mix Percoll and 3×F H_2O to make 40%, 50%, and 60% (v/v) Percoll solutions. Keep on ice.

    b. Place the dissociated cell mixture on the bottom of a 15-ml polystyrene (for greater visibility) conical tube.

    c. Using a baked 9-in. Pasteur pipette, layer 2.5 ml of 40% solution beneath the cell suspension, then carefully dispense the 50% solution under the 40% layer. Finally, carefully dispense the 60% solution beneath the 50% layer. Be sure to dispense all solutions from the tip of the pipette in a slow continuous stream.

13. Centrifuge the gradient in a benchtop centrifuge 10 min at 1800 rpm (~900 × *g*), 4°C.

    The Percoll gradient resulting in the optimum separation of neurons from support cells was determined empirically.

14. Remove tube from the centrifuge and place in a stable rack, taking care not to disturb the gradient. Visually inspect the gradient. Carefully draw off the top myelin-containing layer to reduce contamination of cells banding lower on the gradient. A band of cells should be apparent at the 50%:60% interface. This band will contain highly enriched neurons.

15. Insert a baked 9-in. glass Pasteur pipette into the band of neurons and draw the banded cells into the pipette. Place the Percoll/cell mixture into a 15-ml polystyrene conical tube. Rinse by filling the tube with 3×F H_2O and pelleting the cells by centrifuging in a benchtop centrifuge 10 min at 1800 rpm (~900 × *g*), 4°C.

    A second gradient is not useful unless the first gradient has been overloaded.

16. Decant supernatant and resuspend the pellet in ~12 ml 3×F H_2O. Repeat two additional times.

17. After the final rinse, decant the supernatant and resuspend the pellet in a small volume (e.g., 300 to 500 µl) 3×F H_2O. Transfer resuspended cells to a 1.5-ml centrifuge tube and examine one drop using a microscope. Determine number of neurons (see Support Protocol).

    For examples of results, see Figure 25A.2.2, panels A.2 to C.2, A.3, and B.3.

    Many factors will influence the separation of the cell suspension on the Percoll gradient. Thus, adjusting the gradient to give the separation desired may be required. Monitoring the distribution of cells throughout the gradient is helpful when beginning to determine optimum separation conditions.

18. Analyze DNA or RNA from single cell populations (see Basic Protocols 2 and 3).

SUPPORT PROTOCOL

DETERMINING NUMBER OF NEURONS RECOVERED

In the preceding method (see Basic Protocol 1), there are two steps (i.e., steps 10 and 17) at which evaluating the yields of the cell type of interest should be performed. The following procedure is presented for the evaluation of neurons but can be easily adapted for any cell type that can be distinguished on the basis of morphology or specific marker protein.

Materials

Cell pellet (see Basic Protocol 1)
Cresyl violet solution (see recipe)
95% and 100% ethanol
Xylene
Permount
Superfrost/Plus glass slides (Fisher) or equivalent with coverslips

Additional reagents and equipment for analyzing neuron-specific proteins (e.g., neurofilament 200 kDa peptide) by immunohistochemistry (Sawtell, 1997)

1. Resuspend cell pellet in a known volume of 3×F H_20. Mix tube well by flicking and inverting several times to ensure uniform distribution of cells. Dot five 1-µl aliquots of the cell suspension onto a Superfrost/Plus glass slide or equivalent. Keep cell suspension thoroughly mixed during aliquoting. Dry slide thoroughly.

    If more than one type of assessment is to be performed, multiple slides should be prepared.

2. Stain the slide with cresyl violet solution by overlaying the staining solution onto the slide for 5 min at room temperature, rinsing in deionized water, and dehydrating by dipping once in 95% ethanol and then twice in 100% ethanol. Clear the dehydrated slide in xylene and mount coverslip using Permount.
3. Identify neurons on the basis of morphology using a microscope. Count the number of neurons in each 1-µl aliquot. Determine the average number of neurons per microliter and calculate the total number of neurons by multiplying the average number per microliter by the total number of microliters cell suspension.
4. Analyze an additional slide by immunohistochemistry for a neuron-specific protein, such as neurofilament 200 kDa peptide (detailed in Sawtell, 1997).

   The number of neurons determined by morphology should be similar to that determined on the basis of neurofilament 200 kDa peptide staining.

NONPERFUSION FIXATION WITH STF SOLUTION

ALTERNATE PROTOCOL 1

In some cases, perfusion fixation is not possible. The following procedure is an alternative to perfusion fixation for subsequent analysis of DNA.

Additional Materials *(also see Basic Protocol 1)*

Harvested tissue, fresh
HBSS (see recipe)

1. Finely mince freshly harvested tissue in a drop of STF on a glass slide.

   The tube sizes and volumes given are appropriate for 30 to 40 mg of tissue. If larger amounts of tissue are used, tube sizes and volumes should be scaled up accordingly.

2. Transfer minced tissue to a 1.5 to 2-ml tube containing 1 ml STF and incubate for the desired time at room temperature.

   The optimum fixation time must be determined empirically. In a preliminary experiment, divide minced tissue into several tubes and fix 5 to 15 min.

   Fixation is carried out at room temperature so that subsequent dissociation is possible; therefore, this method is not recommended for separation of cells to be used for downstream analysis of RNA.

3. Following fixation, rinse minced tissue by microcentrifuging tissue 5 min at 5000 rpm, room temperature, then drawing off the supernatant and resuspending the pellet in HBSS. Repeat this process four times.
4. Treat the fixed minced tissue (Basic Protocol 1, steps 7 to 9). Examine the dissociation properties of the cells and the integrity of the nucleic acids and proteins. Select the fixation time yielding good separation and integrity.
5. Proceed as described for perfusion fixation (see Basic Protocol 1, steps 10 to 18).

PREPARATION OF *lacZ*-EXPRESSING CELLS FROM SOLID TISSUES

ALTERNATE PROTOCOL 2

In this example, a procedure used in the author's laboratory, mice expressing an *E. coli* β-galactosidase expression cassette are perfusion fixed using a modification of the procedure described above (see Basic Protocol 1), to visualize *lacZ*-expressing cells.

Materials *(also see Basic Protocol 1)*

Glutaraldehyde
100 µg/ml Xgal in Xgal buffer (see recipe)

1. Perfusion-fix the animal (see Basic Protocol 1, steps 1 to 4), except add 0.2% (w/v) glutaraldehyde to the STF and pump 20 ml of this solution through the animal at room temperature. Proceed with 80°C STF-only perfusion as described (see Basic Protocol 1, step 5).

   This preserves β-galactosidase activity which does not remain active in STF alone. The author has utilized mice infected with a viral mutant containing a β-galactosidase expression cassette; however, mice containing a β-galactosidase transgene or mice in which a β-galactosidase cassette has been introduced using any gene transfer approach could also be analyzed in this way.

2. Remove tissue of interest and incubate in 100 µg/ml Xgal in Xgal buffer at 37°C for 3 hr.

   The time of incubation in the Xgal will depend on the strength of the promoter driving expression. The minimum amount of time for development should be used.

3. Inspect tissue and confirm presence of "marked" cells, then mince and dissociate the tissue (see Basic Protocol 1, steps 6 to 9).

4. Enrich cell population by Percoll gradient separation (see Basic Protocol 1, steps 10 to 17) or other suitable method.

   Blue neurons are enriched in the bottom of the gradient, presumably because of increased density from the precipitated X-gal reaction product. This is shown in Fig. 25A.2.1E.1 to 3.

BASIC PROTOCOL 2

ANALYSIS OF SINGLE CELLS BY PCR

In the following section, a protocol for analyzing the dissociated enriched neurons by PCR to detect the HSV thymidine kinase gene is presented; however, this protocol can be applied to other cell types and nucleic acids as well. The goal in developing this assay was to provide a method for the quantitative assessment of the number of neurons containing the HSV genome. Because the frequency of the latent viral genome in the author's experimental system was relatively high (20% to 30% of the total neurons in the ganglion), the analysis had to be performed on single neurons; however, depending on the frequency of the nucleic acid of interest in the cell pool being analyzed, it could be possible to perform the analysis on samples containing groups of known numbers of cells.

The primers and basic PCR conditions are essentially as reported by Katz and Coen (1990) and detailed in *UNIT 15.7*. Steps are included here for (1) aliquoting cells, (2) confirming the number of cells per tube being analyzed, and (3) eliminating any extracellular contaminating DNA. This step is critical to ensure that the DNA being amplified is actually intracellular. This is done by using DNase linked to beads. The bead cannot enter the cell, and thus the DNase is able to digest DNA in the fluid surrounding the cell, but does not destroy the DNA within the cell. In the next steps, which include a proteinase K treatment (to increase the permeability of the cell) and the PCR reaction itself, a two-part buffer system is utilized to minimize pipetting and insure maximum uniformity in the setup of samples by eliminating the need to pipet very small volumes.

Materials

Enriched cell sample (see Basic Protocol 1 or Alternate Protocols 1 or 2)
Triple 0.2-µm filtered nanopure (3×F) H_2O
Ponceau S solution (see recipe)
Immobilized-DNase on PVP beads (Mobitec)
DNase reaction buffer (see recipe)
PCR/PK solution (see recipe)

DNA standards—e.g., cloned segments of HSV genome containing the gene being amplified (e.g., thymidine kinase)
PCR amplification solution (see recipe)
Taq DNA polymerase (Life Technologies)

200-µl PCR tubes
Dissecting microscope
PCR Gene Amp 2400 (Perkin Elmer Cetus)
Gene screen plus nylon membrane (NEN Life Science Products)
Storage phosphor screen (Molecular Dynamics)
Imagequant software

Additional reagents and equipment for quantitating standards (*UNIT 15.7*; Sawtell and Thomson, 1992), PCR (*UNIT 15.1*), nondenaturing polyacrylamide gel electrophoresis (*UNITS 2.5 & 2.7*), UV-crosslinking DNA to filters (*UNIT 2.9*), hybridizing blots with oligonucleotides (*UNITS 2.9A & 6.4*), labeling oligonucleotides (*UNITS 4.6, 4.8 & 15.7*), and phosphorimaging (*APPENDIX 3A*)

Select single neurons

1. Dilute a portion of the enriched neuron sample with 3×F H_2O so that 1 µl contains ~1 neuron. Add Ponceau S solution to a final volume of 1/200 and aliquot 1 µl neuronal suspension into the bottom of a 200-µl PCR tubes.

   This dye allows easy visualization of neurons in the bottom of the PCR tube when viewed under a dissecting microscope, but does not interfere with subsequent analyses.

2. Examine each tube under a dissecting microscope and identify those containing a single neuron for use in step 3.

   The number of tubes will depend upon the anticipated frequency of the DNA sequence being analyzed. In the authors studies, a typical analysis will include 200 single neuron samples.

Immobilized DNase treatment and PCR reaction

3. Resuspend immobilized-DNase on PVP beads in DNase reaction buffer so that 5 µl contains ~100 beads. Add a 5-µl aliquot to each PCR tube containing a single neuron. Mix gently. Incubate samples several hours or overnight at 37°C.

   The purpose of the DNase treatment is to make sure that the DNA being measured is the DNA within the cell or cells in the PCR tube. While DNase treatment could be performed on cells en masse, one could not be sure that some cells were not broken during purification and aliquoting of cells.

   Prior to use of the immobilized DNase, it is important to confirm that the DNase activity in the preparation is associated with the bead, and that no free DNase activity can be detected, as any free DNase could enter the cell and destroy the intracellular DNA. To test this, the immobilized DNase in activation buffer is pelleted gently (as detailed by the manufacturer) and an aliquot of the supernatant is drawn off and placed in a 1.5-ml microcentrifuge tube. The supernatant is then spiked with intact plasmid DNA of known size and incubated for ~1 hr at 37°C. Agarose gel electrophoresis (UNIT 2.5A) is then used to evaluate integrity of DNA incubated with and without supernatant. The supernatant-treated DNA should show no evidence of degradation.

   When aliquoting cells at the single-cell level, many of the tubes contain no cells. Some of these samples, as well as samples spiked with HSV DNA, are utilized as controls to test the completeness of the DNase treatment (see Critical Parameters and Troubleshooting). The ability of the immobilized DNase to eliminate potential contamination should be determined by spiking a sample with the DNA sequence being amplified. It is important to spike the sample with an amount of DNA that would reflect anticipated levels of contamination. With proper technique, these levels should be extremely low and not present in every cell sample. The DNase step is an important safeguard, but not a solution for poor technique.

4. Place samples in a PCR Gene Amp 2400 or equivalent and heat to 94°C for 5 min to inactivate DNase. Reduce temperature to 50°C, add 34 µl PCR/PK solution to each sample, and incubate 3 hr.

5. Prepare standards and quantitate as described (*UNIT 15.7*; Sawtell and Thompson, 1992). Prepare standard dilutions representing 10,000, 1,000, 100, 10, and 0 HSV viral genomes in 6 µl.

   Standards are treated identically to the cell samples with the exception of DNase treatment. To quantify other nucleic acids of interest, use appropriate standards and optimized PCR assays (UNIT 15.1).

6. Incubate samples and standards 7 min at 94°C to inactivate proteinase K.

7. Incubate at 63°C while adding 10 µl PCR amplification solution and 1.25 U *Taq* DNA polymerase per reaction (50 µl total).

8. Amplify using the following program parameters:

45 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	30 sec	72°C	(extension)
Final step:	7 min	72°C	(extension/hold).

   PCR conditions should be optimized for the primer/target of interest as described in UNIT 15.1.

9. Electrophorese 5 µl each PCR product through a nondenaturing 12% polyacrylamide gel (*UNITS 2.5A & 2.7*), transfer to a Gene screen plus nylon membrane (*UNIT 2.9A*), and perform hybridization analysis (*UNIT 2.9A & 6.4*) using a ^{32}P-end-labeled oligonucleotide internal to the PCR primers (*UNIT 15.7*).

10. Expose blot to a storage phosphor screen (*APPENDIX 3A*) and analyze using Imagequant software.

BASIC PROTOCOL 3

ANALYSIS OF ENRICHED CELL POPULATIONS BY RT-PCR

Presented in this section is a protocol that can be adapted to examine either specific or general transcriptional patterns in groups of selected populations of cells harvested from solid tissues. The cells in the tissue are first stabilized by fixation, avoiding the transcriptional changes that would occur with the manipulation and dissociation of living cells. Using carefully screened reagents, it is possible to maintain the integrity of the RNA within the cells during the dissociation process so that RT-PCR analysis is possible (Sawtell, 1997). The goal of the author was to analyze the RNA contained within just a few cells using RT-PCR. In PCR analysis (see Basic Protocol 2), the integration of the pretreatment steps and the PCR reaction in a single assay tube was straightforward; however, in the case of RT-PCR, establishing the compatibility of all of the enzymatic steps required for the pretreatment, reverse transcription, and subsequent PCR without an extraction step was more challenging. The assay developed is presented below. This protocol has been successfully utilized to detect transcripts in samples of fewer than ten neurons. This approach has proven to be especially useful to examine cell type specific expression of transcripts within solid tissues. For example, the author used this approach to demonstrate that the expression of a novel stress-induced spliced form of a key transcription factor was restricted to the neurons in the trigeminal ganglion (unpub. observ.).

Primer selection will depend on the transcript of interest. The MacVector PCR primer selection program has proven to consistently yield primers that work well. If specific

transcripts are being analyzed, primers that span splice sites is a distinct advantage. If a nonspliced transcript is being amplified, it is imperative to include sufficient controls in which the reverse transcriptase has been omitted to rule out the possibility that DNA rather than RNA is being amplified. One limiting factor will be the length of product generated by the reverse transcription reaction. The author has had success using this direct fixed cell RT-PCR assay with primers to mouse genes that generate a 500-bp product.

Materials

Proteinase K solution (see recipe)
40 mM PMSF, fresh
RNase-free DNase I: 3 U RNase-free DNase I (Boehringer Mannheim)/25 mM DTT/ 2.5 U placental RNase inhibitor
8 pmols/µl reverse transcriptase primer
Reverse-transcription reaction mix (see recipe)
200 U/µl SuperScript II reverse transcriptase (Life Technologies)
PCR amplification solution (see recipe)
1.25 U *Taq* DNA polymerase (Life Technologies)
PCR tubes

Additional reagents and equipment for obtaining dissociated perfusion-fixed cells (see Basic Protocol 1) and PCR optimization (*UNIT 15.1*)

1. Obtain cells dissociated from perfusion fixed tissues as detailed above (see Basic Protocol 1) using solutions tested to be free of RNase activity.

   At this point, immobilized RNase could be utilized to remove any contaminating RNA from the aliquoted cells, as detailed above (see Basic Protocol 2, step 3); however, the author has tested extensively for specific RNAs in the supernatant of washed, dissociated cells and has not detected extracellular RNA contamination. This could reflect the inability of the reverse transcription reaction to detect one or just a few template molecules. In contrast, HSV DNA could occasionally be detected in the supernatant; therefore, eliminating it was imperative.

2. Aliquot cells to be analyzed in a 1-µl volume into PCR tubes. Add 4 µl proteinase K solution and incubate 60 min at 50°C. After digestion, add 0.25 µl freshly prepared 40 mM PMSF.

   Preliminary analysis demonstrated the need for protease digestion of cellular proteins in isolated cells for complete DNase I digestion of genomic DNA; however, the high temperatures required for heat inactivation of this enzyme led to degradation of RNA, most likely through metal ion-catalyzed hydrolysis. Thus, following digestion with proteinase K, activity of this enzyme is selectively inhibited by adding freshly prepared PMSF.

3. Add 0.75 µl RNase-free DNase I. Incubate 45 min at 37°C.

4. Inactivate DNase by incubating 15 min at 70°C. After this time, add 0.25 µl of 8 pmol/µl (2 pmol total) reverse transcriptase primer and incubate an additional 10 min at 70°C.

5. Reduce temperature to 50°C and add 3.5 µl reverse-transcription reaction mix, followed by an additional 0.25 µl of 40 mM PMSF and 0.25 µl of 200 U/µl (50 U) SuperScript II reverse transcriptase. Incubate 60 min at 50°C.

   If the transcripts are to be detected are unspliced, samples are set up in multiples, half of which receive no reverse transcriptase. In additional controls, RNase is included with the DNase (step 3).

6. After 60 min, increase temperature to 70°C for 15 min. Add $\frac{1}{5}$ to $\frac{1}{2}$ of the cDNA sample to 47 µl PCR reaction buffer and heat 5 min at 94°C.

7. Reduce the temperature to 5°C above the annealing temperature (*UNIT 15.1*) and add 1.25 U *Taq* DNA polymerase to each sample (50 µl total).

8. Analyze amplification products as described above (see Basic Protocol 2, steps 9 and 10)

The usefulness of the RT-PCR assay for quantification at the single-cell level has not been fully explored. Using primers to specific HSV genes, the reverse transcription reaction lacked the sensitivity required to detect the very low levels of these transcripts anticipated during viral latency. This may be due, in part, to the very high GC content of the HSV genome in general and the specific regions being reverse transcribed. Regardless, the assay can detect specific transcripts in small numbers of cells.

REAGENTS AND SOLUTIONS

Use 3×F H_2O in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Cresyl violet solution

Prepare the following in triple 0.2-µm filtered nanopure (3×F) H_2O:
0.5% (w/v) cresyl violet
10% (v/v) glacial acetic acid
Store up to 12 months at room temperature

DNase reaction buffer

Prepare the following in triple 0.2-µm filtered nanopure (3×F) H_2O:
20 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
5 mM $MgCl_2$
5 mM $CaCl_2$
Aliquot and store up to 12 months at −20°C

Hanks balanced salt solution (HBSS)

0.4 g/liter KCl
0.06 g/liter KH_2PO_4
8.00 g/liter NaCl
0.35 g/liter $NaHCO_3$
0.048 g/liter Na_2HPO_4
1.00 g/liter D-glucose
Sterilize by passing through three 0.2-µm filters
Aliquot and store up to 12 months at −20°C

PCR amplification solution

Prepare the following in triple 0.2-µm filtered nanopure (3×F) H_2O:
20 mM Tris·Cl, pH 8.4 (*APPENDIX 2*)
50 mM KCl
1.5 to 4.5 mM $MgCl_2$
5% (w/v) gelatin
200 µM each dNTP
25 to 50 pmols of each primer (*UNIT 15.7*; Katz et al., 1990)
Store up to 1 month at −20°C

While the buffer can be stored with primers and dNTPs, it is better to add them just before use. Buffer without primers or dNTPs can be stored up to 12 months at −20°C.

The concentration of $MgCl_2$ will depend on specific primers utilized (UNIT 15.1) but will commonly range between 1.5 to 4.5 mM.

PCR/PK solution

Prepare the following in triple 0.2-µm filtered nanopure (3×F) H_2O:
20 mM Tris·Cl, pH 8.4 (*APPENDIX 2*)
50 mM KCl
1.4 to 4.5 mM $MgCl_2$
Aliquot and store up to 12 months at −20°C
Just before use, add 50 µg/ml proteinase K

The concentration of $MgCl_2$ will depend on specific primers utilized (UNIT 15.1) but will commonly range between 1.5 to 4.5 mM.

Ponceau S solution

Prepare the following in triple 0.2-µm filtered nanopure (3×F) H_2O:
0.5% (w/v) Ponceau S
1% (v/v) glacial acetic acid
Store in aliquots up to 12 months at room temperature.

Proteinase K solution

25 mM Tris·Cl, pH 8.4 (*APPENDIX 2*)
37 mM KCl
1.5 mM $MgCl_2$
0.3 µg proteinase K
Make fresh

Reverse-transcription reaction mix

93 mM Tris·Cl, pH 8.3 (*APPENDIX 2*)
140 mM KCl
5.5 mM $MgCl_2$
Store up to 12 months at −20°C
Just before use add DTT to 25 mM and dNTPs (*UNIT 3.4*) to 0.25 mM

Xgal buffer

Prepare the following in "clean" phosphate buffered saline, pH 7.4 (PBS; *APPENDIX 2*):
5 mM $K_3Fe(CN)_6$ (potassium ferrocyanide)
5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ (potassium ferricyanide)
2 mM $MgCl_2$
Aliquot and store up to 3 months at room temperature

COMMENTARY

Background Information

The concept of "cellular pathology" was put forth nearly 150 years ago by Virchow (1863) with the view that disturbances in structure and function of individual cells form the basis of disease. Current understanding of the interactive nature of the cells comprising an organism have substantiated this view. It is now clear that cells differentiate and function according to the summation of the molecular cues arising from many other cells in the organism (Gilbert, 1994). It follows that certain important aspects of the molecular behavior of individual cellular components can only be observed in the context of the organism.

Reported here is a strategy, contextual expression analysis (CXA), that combines the cell-specific information of in situ approaches with the analytical and quantitative potential of solution PCR (Sawtell, 1997). Cells are chemically stabilized in the context of the organism and subsequently isolated. PCR can then be utilized to gain insight into the molecular processes of a single cell among billions.

The enzymatic dissociation of living tissues has been widely used and refined for many specific tissue types (Pretlow and Pretlow, 1982). Inherent in this process are cellular molecular changes induced by disruption of context. In order to prevent these changes, tissues

are stabilized by chemical fixation prior to dissociation. Yields of even fragile adult cell types such as neurons are high. Distinct morphological features, such as brush borders of the intestinal epithelial cells as well as nuclear and cytoplasmic nucleic acid staining patterns are comparable to those in sectioned tissues (Sawtell, 1997). DNA and RNA isolated from dissociated tissues are reasonably intact. By immunohistochemical staining, the distribution of cytoskeletal proteins including actin and neurofilament 200 kDa peptide are similar in dissociated cells when compared to sectioned tissue (Sawtell, 1997).

The impetus for development of this protocol arose out of the need in the author's laboratory to identify the cell types and to quantify the number of cells in a specific solid tissue that harbored the latent HSV genome. It was also important to determine the number of viral genomes in each of those cells. The approach has proven extremely useful for this purpose (Sawtell, 1997; Thompson and Sawtell, 1997, 2000, 2001; Sawtell et al., 1998; Sawtell et al., 2001); however, this method should be widely useful for facilitating the analysis of rare cells or cellular events occurring in a complex multicellular environment.

Critical Parameters and Troubleshooting

In developing this procedure, several common fixation formulations were tested in combination with alternative digestive enzymes. For the most part, tissues either remained a solid mass or disintegrated into cellular debris; however, perfusion with STF followed by digestion with collagenase (i.e., Worthington CLSI) yielded single cell suspensions from peripheral and central nervous tissue, lung, liver, intestine, heart, pancreas, muscle, and reproductive tract. Nonetheless, optimizing the balance between fixation and dissociation for the specific tissue of interest is advisable.

Fixation

The volume of fixative perfused through the animal is critical and should be measured. The procedure can be modified for larger animals, such as guinea pigs, by increasing the volume of fixative utilized.

Several different types of fixatives were tested, including various formaldehyde based formulations. The fixative found to give the best results was Streck tissue fixative (STF). This is a noncrosslinking fixative containing diazolidinyl urea, 2-bromo-2-nitropropane-1,3-diol (bronopol), zinc sulfate, and sodium citrate. Why STF works in Basic Protocol 1 has not been explored; however, it is likely that absence of crosslinking in the fixed tissue is favorable for the subsequent enzymatic dissociation process.

Mincing

It is critically important to finely and uniformly mince the tissue. This allows greater and more uniform access of dissociating enzymes to the tissue.

Collagenase

The collagenase preparation used contains several collagenases, as well as caseinase, clostipain, and tryptic activities. This is a relatively crude preparation and there is lot-to-lot variation in this product. The author's laboratory has tested a number of purified enzymes with varying levels of success; however, none worked as well as the crude preparation. Batches of collagenase must be screened not only for optimum dissociation activity but also for DNase and RNase activity, depending upon final use of cells. This can be done easily by spiking an aliquot of the enzyme (5× strength) with DNA or RNA. The sample is then incubated at 37°C for 1 hr and examined by agarose gel electrophoresis for integrity of the nucleic acids. While many batches will be free of DNase, RNase-free collagenase is less common.

Contamination

Careful planning to prevent contamination control is necessary for the success of this procedure. *UNIT 15.7* discusses many of the relevant issues. It is critical that the results obtained from the PCR be related to the contents of the cell being analyzed and not contamination introduced at any point during the procedure. With all aspects of this procedure, the final use of the cells will determine the types of contamination that must be avoided. The most meticulous technique is required if downstream applications require intact RNA. The introduction of RNases at any point must be avoided.

Controls

Observe the tissue and cells of interest frequently during the dissociation process. Such observation will provide important information regarding the response of the dissociating tissue and cells to the process. Make estimates of the

number of cells expected and determine if recoveries are reasonable. There should be minimal cell loss with this method.

Examine the integrity of the nucleic acids and proteins by carrying out routine isolation and analytical procedures on the tissue after dissociation.

All standard PCR and RT-PCR controls should be included. Additional controls will be required, depending on the final application of the cells.

Anticipated Results

Single cell suspensions of tissues will be obtained. With proper attention to the quality of reagents used, specifically the collagenase, the nucleic acids within the cells will be intact. Keep in mind that the results obtained from downstream analyses will depend on the quality of the cells obtained from the dissociation step.

Time Considerations

The time required will depend on the skill level of the individual performing the task. For the skilled practitioner, mouse perfusion will require 10 to 15 min, dissection and mincing of tissue 5 to 15 min (depending on how many tissues are being dissected), tissue dissociation 30 to 45 min, rinsing and post-fixation 20 min, neuron counts ~1 hr (depending on how many slides are examined), separation of Percoll gradient and rinsing 1 hr, aliquoting individual cells one to several hours (depending on the number of tubes), PCR (including all pretreatments) 1.5 to 2 days (this is not a continuous effort), and gel electrophoresis, prehybridization, and hybridization 1 to 1.5 days (this also is not a continuous effort).

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Key References

Sawtell, N.M. 1997. See above.

This manuscript describes the procedure as used to quantify viral latency and includes several critical validation experiments.

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Laser Microdissection-Mediated Isolation and In Vitro Transcriptional Amplification of Plant RNA

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ABSTRACT

Protocols for laser microdissection and linear amplification of RNA from fixed, sectioned plant tissues are described. When combined with quantitative RT-PCR, microarray analysis, or RNA-sequencing, these procedures enable quantitative analyses of transcript accumulation from microscopic quantities of specific plant organs, tissues, or single cells. *Curr. Protoc. Mol. Biol.* 87:25A.3.1-25A.3.15. © 2009 by John Wiley & Sons, Inc.

Keywords: laser microdissection • plants • RNA amplification • transcriptomics

INTRODUCTION

This unit describes a method for the isolation of RNA from plant structures using laser microdissection (LM). LM technology permits precise isolation of specific tissues, organs, or cells from fixed and sectioned plant tissues adhered to microscope slides. In many cases, the quantity of sample material isolated by LM is limiting. However, nanogram quantities of RNA extracted from microdissected plant tissue—or any RNA sample—can be linearly amplified using T7 RNA polymerase (Luo et al., 1999) to generate microgram quantities of RNA. This microdissected amplified RNA (aRNA) is subsequently used as a template for the preparation of cDNA, which can then be utilized in a variety of transcriptomic analyses including quantitative RT-PCR (qRT-PCR), microarrays hybridization, or massively parallel sequencing (RNA-Seq). The power and allure of LM technology for plant biological research lies in the ability to sample discrete microdomains or cell types within plant tissues, thereby eliminating the transcriptional background noise contributed by adjacent or contaminating unrelated tissues. In this way, profiles of localized gene expression are generated that are resolutely focused on the cells and tissues of interest (Fig. 25A.3.1). The protocols described in this unit are adapted specifically for microdissection of plant cells and tissues, whose properties (including cellulosic cell walls and large hydrolytic vacuoles) present unique challenges to the implementation of LM technology. Accordingly, the LM protocol described here differs from those in *UNITS 25A.1 & 25B.8*, which are optimized for animal cells and tissues. For additional reviews on the use of LM for transcriptional profiling in plants, see Kehr et al. (2003), Day et al. (2005), and Nelson et al. (2006).

A variety of laser-assisted microdissection platforms are commercially available; users are advised to evaluate several systems before deciding which platform is best suited for their samples. The authors' laboratories currently use the PALM (P.A.L.M. Microlaser Technologies, Carl Zeiss) laser microdissection and pressure catapulting system (LMPC), in which a pulsed ultraviolet (UV-A) laser beam cuts cells from tissue sections and laser pressure is used to catapult these selected tissues into collection caps

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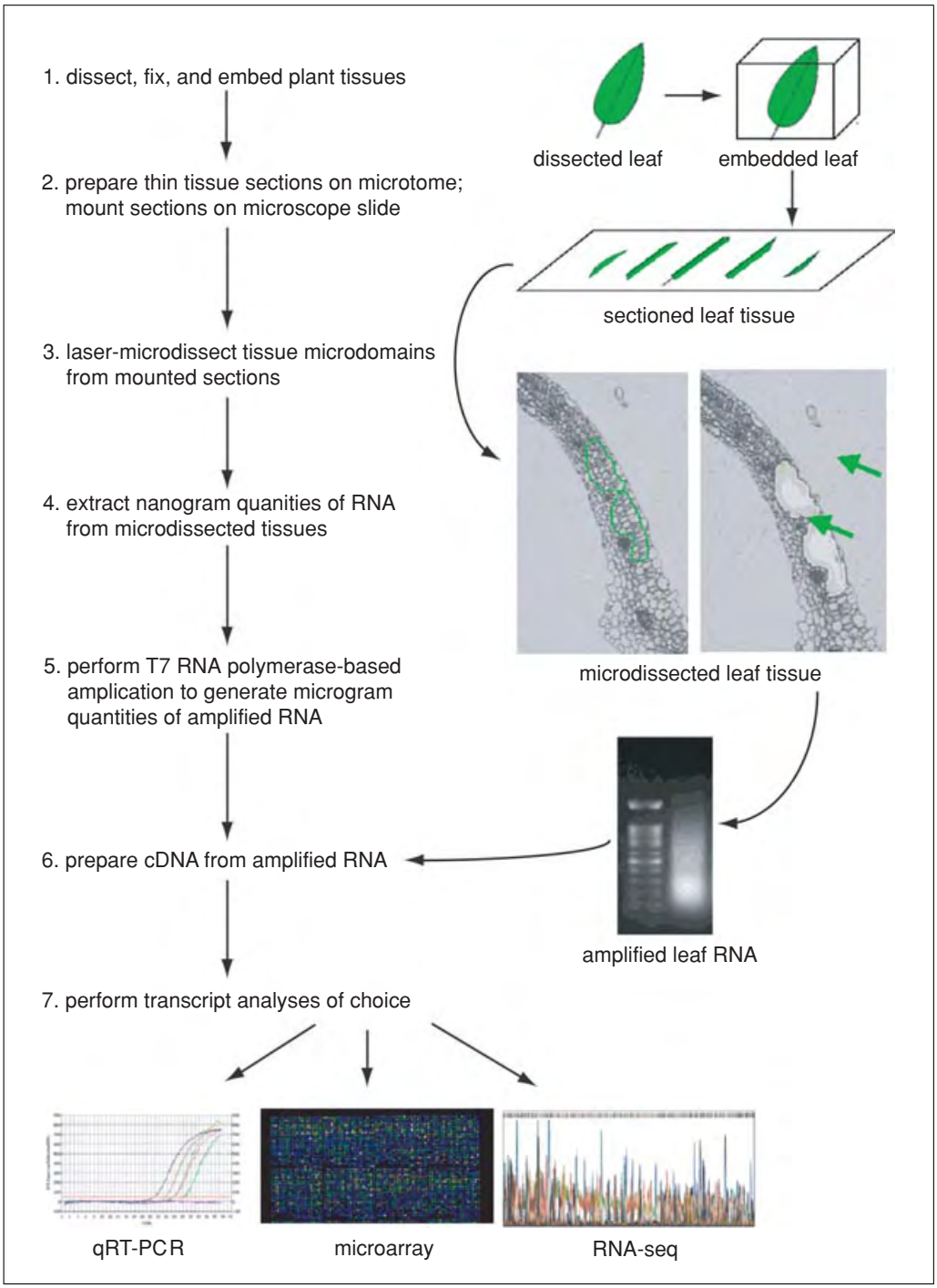
1. dissect, fix, and embed plant tissues
dissected leaf
embedded leaf
2. prepare thin tissue sections on microtome; mount sections on microscope slide
sectioned leaf tissue
3. laser-microdissect tissue microdomains from mounted sections
4. extract nanogram quanities of RNA from microdissected tissues
5. perform T7 RNA polymerase-based amplication to generate microgram quantities of amplified RNA
microdissected leaf tissue
6. prepare cDNA from amplified RNA
amplified leaf RNA
7. perform transcript analyses of choice
qRT-PCR
microarray
RNA-seq

Figure 25A.3.1 Flowchart of the use of laser microdissection for analysis of transcript accumulation within plant tissue microdomains. In this example, mesophyll cells are microdissected (green arrows) from transverse sections (10-μm) of mature rosette leaves of *Arabidopsis thaliana.* Images of *Arabidopsis* leaf sections were provided by K. Petsch, Cornell University; agarose gel image of IVT-amplified RNA is kindly provided by X. Zhang, University of Georgia.

mounted above the samples. Thus, the PALM system enables the destruction of closely surrounding, non-targeted tissues by laser ablation *before* isolation of the cells/tissues of interest, thereby eliminating undesired contaminant transcripts from the sample pool.

LASER MICRODISSECTION OF PLANT RNA

The following protocol details procedures for LM and subsequent RNA isolation/amplification from acteone-fixed and paraffin-embedded shoot apical meristem (SAM) tissue obtained from 14-day-old maize seedlings. Although procedures are focused on SAM tissue, this method has been utilized in LM analyses of a variety of plant cell and tissue types with minor modifications in infiltration and embedding times, as described below.

Materials

Maize seedlings 14 days post-germination
Acetone (100%, Fisher Scientific), ice-cold and room temperature
Ice
Xylene (Fisher Scientific)
Diethylpyrocarbonate (DEPC; Sigma)
100% ethanol
Mineral oil (optional)
PicoPure RNA Isolation Kit (Arcturus)

Razor blade (single-edged)
Petri dishes (glass)
Scintillation vials (20 ml, Fisher Scientific)
Vacuum apparatus
Rotator (e.g., Ted Pella)
Paraplast chips (Paraplast +, 56°C, Oxford Labware)
Oven preset to 60°C
Gradient metal warming plate (a paraffin-embedding center can be used if one is available)
Metal weighing dish
Tweezers *or* paintbrush (fine point)
Paraffin embedding rings (Simport)
Paraffin clear base molds (Surgipath)
Plastic bags
Rotary microtome
Probe-on-Plus slides (Fisher Scientific) *or* PEN Membrane Slides (P.A.L.M. microbeam)
Slide-warming tray (Fisher Scientific)
Paper towels
Dissecting microscope
PALM MicroBeam System (Carl Zeiss)
PALM adhesive cap tubes (Carl Zeiss) or 0.5-ml centrifuge tubes with caps

NOTE: Work in a fume hood until samples are securely capped and placed at 4°C. Keep fixative cold at all times to ensure slow penetration of fixative.

Fix samples

1. Using a fresh single-edged razor blade, separate the seedling shoot from the root by slicing at the coleoptile node, which is the point of insertion of the first leaf-like organ of the shoot. Retain the apical portion (i.e., the shoot) and place in a glass petri dish containing ice-cold acetone. Immediately execute a second cut ~1 cm above the coleoptile node and retain the lower portion, which will isolate the base of the shoot containing the SAM from the upper portion of the shoot containing expanded leaves.

   IMPORTANT NOTE: *High concentrations of acetone can cause dizziness, confusion, unsteadiness, and unconsciousness if it comes into contact with the lungs, digestive tract, or skin. Wear gloves and always work in a fume hood.*

This step results in an ~1-cm long segment of maize seedling shoot tissue that contains a short segment of stem topped by the SAM, which is surrounded by basal portions of approximately 14 leaves or whole leaf primordia.

2. Trim tissues while submersed in acetone fixative to a final size of ~0.3 × 0.3 × 0.2 cm. Place them in a scintillation vial with 15 ml of ice-cold 100% acetone and keep on ice. Prepare eight to ten seedlings in this manner and place into the same vial; the volume ratio of fixative to sample should not be less than 20:1. Work rapidly.

   The total preparation time for a single vial of eight to ten seedlings should not exceed 10 min.

   Trimming of seedlings in the manner described above will ensure that the SAM is retained in sample blocks that are also small enough to permit two rows of samples to be mounted on a single slide (see below). For larger tissue samples, be sure to trim samples to a size small enough to be mounted on a 25 × 75–mm microscope slide.

3. Vacuum infiltrate the samples (on ice) by subjecting the vial to a vacuum of 400 mmHg for 10 to 15 min. Slowly equilibrate to atmospheric pressure to avoid bumping or boiling the solution. Decant acetone and replace with fresh ice-cold 100% acetone. Re-cap the vial and allow samples to fix overnight at 4°C on a rotator.

   Vacuum infiltration is required to remove air spaces trapped within samples that may prevent penetration of fixative. If the samples sink readily, this step may not be necessary. Be careful not to boil the solution during vacuum infiltration, as this may cause tissue damage. As air is removed, the samples will rise to the surface; infiltrated samples will rapidly sink to the bottom of the vial once the vacuum is released.

Xylene infiltrate samples

4. The next day, bring the samples to room temperature and replace the fixative with fresh room-temperature 100% acetone. Rotate 1 hr at room temperature.

5. Replace the fixative with a mixture of acetone:xylene (1:1) and rotate 1.5 hr at room temperature.

6. Perform three solution changes of pure xylene, incubating 1 hr at room temperature after each change.

   IMPORTANT NOTE: *Xylene is irritating to the skin, eyes, and respiratory tract, ingestion or inhalation can cause systemic toxicity. Always work in a fume hood.*

   Acetone is a polar yet versatile solvent that is miscible with H_2O, as well as most nonpolar organic solvents. During the fixation step, all aqueous components of the tissue are gradually replaced with the polar solvent acetone. During xylene infiltration, the acetone is gradually replaced with the nonpolar solvent xylene in preparation for embedding with the nonpolar Paraplast medium. If the sample is very dense or contains multiple tissue layers, more gradual infiltration should be performed using 3:1, 1:1, and 1:3 mixtures of acetone:xylene.

Perform paraffin infiltration and embedding of samples

7. Add a small amount (~1/10 to 1/5 the volume of the vial) of Paraplast chips to each vial and incubate overnight at room temperature on a rotator.

   From this point on, care should be taken to avoid introduction of RNases during handling of samples.

8. The next day, incubate a separate container of Paraplast chips at 60°C for several hours or until completely melted. At the same time, place the vials containing the plant tissue in an oven at 60°C to dissolve any remaining Paraplast chips. When chips are dissolved, gently invert the vials to mix xylene and Paraplast.

   The temperature of the molten Paraplast (60°C) is critical—overheating will shrink the paraffin and cause tissue damage.

9. Incubate the vials containing the plant tissue for an additional 1.5 hr at 60°C, add more Paraplast chips (up to half the volume of the vial), and incubate for an additional 1.5 hr at 60°C.

10. Carefully decant half the volume of the xylene/Paraplast mixture and discard while ensuring that the tissue samples remain in the vials. Replace the decanted solution with 60°C molten 100% Paraplast from step 8. Mix by inversion, and incubate overnight at 60°C. Be sure to maintain a container of 60°C molten, 100% Paraplast for use in steps 11 to 14.

11. The next day, decant the contents of the vial into a waste receptacle, being careful not to dispose of any tissue samples. Replace the volume with 60°C molten 100% Paraplast, and incubate at 60°C. Renew with fresh molten Paraplast twice per day (at the beginning and end of each workday).

12. Repeat Paraplast infiltration at 60°C (as in step 11) for 4 additional days. Incubate overnight at 60°C after the last Paraplast change.

13. Prepare a gradient metal warming plate that is hot enough to melt Paraplast at one end (but no greater than 60°C), and room temperature on the opposite end (a commercially available paraffin-embedding station can be utilized for greater convenience).

14. Pour tissue and Paraplast from the vial into a metal weighing dish that has been placed on the hot side of the warming plate. Using tweezers or a small paintbrush that is dedicated for use with molten Paraplast, carefully transfer a single-shoot tissue, along with some molten Paraplast, into the base mold. Orient the tissue in the proper position for microtome sectioning, and place the paraffin-embedding ring over the base mold. Fill the embedding ring/base mold assembly (block) with molten paraffin. Cool the block to room temperature slowly (over at least 20 min), by sliding gradually further and further down the warming plate toward the cool end. Store solidified blocks in plastic bags at 4°C.

    Overheated Paraplast will shrink and can cause tissue damage; do not exceed 60°C. Denser materials will require longer Paraplast infiltration times. Incompletely infiltrated tissues cannot be properly sectioned, and may tear or break free of Paraplast blocks.

    Embedded tissue samples must not settle to the very bottom or edge of the base mold. To avoid tissue breakage during sectioning, samples should be completely surrounded by Paraplast at least 2-mm thick.

Perform microtome sectioning

15. Trim Paraplast blocks into a trapezoidal shape ensuring that the top and bottom edges of the block are parallel to each other, and to the edge of the microtome knife blade. Section the blocks on a rotary microtome (typically 10-μm sections are used).

    The individual tissue sections will remain attached to both the preceding and the subsequent sections, to form a serial ribbon of tissue sections that can be handled using fine-pointed paintbrushes.

    Do not handle tissue ribbons with the fingers; the heat generated by the human hand is sufficient to partially melt the paraffin sections, which will adhere to the fingers if handled.

    Microtome sectioning causes considerable tissue compression. Allow for ~25% ribbon expansion in all dimensions when cutting ribbons to place on slides, in order to prevent overcrowding of sections. For helpful hints on tissue sectioning techniques and troubleshooting, consult "Plant Microtechnique and Microscopy" by S. Ruzin (1999).

16. Use a razor blade to trim ribbons containing sectioned samples of interest to fit onto microscope slides. Using a fine-pointed paintbrush, carefully place tissue sections

onto slides floated with DEPC-treated water. Place slides with sections onto a slide-warming tray at 40°C for ~5 min, or until the sections relax and decompress (not to exceed 20 min).

Probe-on-Plus slides are coated with a charged tissue adhesive and are RNase-free, and thus are convenient and suitable for most LM applications. However, the use of PEN membrane-coated slides permits sample microdissection with a minimal amount of tissue fragmentation.

17. Carefully remove water from underneath the relaxed section ribbons by tipping the slide onto absorptive paper towels. Wick off residual water with tissue paper, being careful not to disturb the ribbons. Quickly place the slide back onto the slide-warming tray and incubate overnight at 40°C to adhere tissue to slides. Be sure to elevate one end of the slide during incubation, to allow for air circulation and prevent the formation of air bubbles beneath the tissue.

    Dried slides can be used right away, or stored in a vacuum desiccator at 4°C for at least 14 days until utilized for laser microdissection.

Perform laser microdissection-microcatapulting of plant cells

18. Bring sample slides to room temperature. Deparaffinize tissue by incubation in two changes of xylene (10 min each), followed by one wash in 100% ethanol (2 min). Air-dry the slide and place onto the microscope stage to mark tissue domains to be collected. Keep remaining slides in 100% ethanol until needed.

19. Prior to sample microdissection, optimize the energy and focus of the laser for the sections that include target cells. Test and optimize the PALM capturing settings for each tissue type.

    To prevent tissue damage, always utilize the minimal laser energy that is required to cut and catapult the tissue from the slide. Cell wall thickness can vary greatly among different cell types, and is a common barrier to successful laser microdissection of plant cells. Laser settings MUST be optimized to each tissue type.

20. Mark areas of target cells using the PALM sample selection software (see Video 1 at *http://www.currentprotocols.com*).

21. Harvest targeted cells into the adhesive cap of the collection tubes via the "Close and Cut plus AutoLPC" method according to the vendor's manual. As an alternative to adhesive caps, samples can be microcatapulted into the cap of a standard 0.5-ml centrifuge tube containing a drop of mineral oil as a tissue adhesive. The mineral oil will not inhibit RNA extraction, described below.

    The focused laser first cuts the outline of the target cells to isolate the tissue of interest from surrounding tissues. Subsequently, the defocused laser catapults the targeted cells into the tube adhesive cap (see Video 1 at http://www.currentprotocols.com).

    Be certain that tissues are laser microdissected at the same magnification as they are marked with the sample selection software, or tissue targeting will be imprecise.

    PALM adhesive caps are coated with an RNase-free tissue adhesive that prevents tissue loss due to fallback from the cap. Be careful not to saturate the cap surface during prolonged laser microdissections. Overfilled caps will no longer adhere to harvested tissue, which may fall back to the slide surface (an unfortunate phenomenon sometimes referred to as "snowing," and which can be easily remedied by inserting a fresh adhesive cap).

22. Collect a sufficient amount of tissue for downstream applications.

    Tissue collected from six to ten maize SAMs typically yields between 5 and 10 ng of RNA, utilizing RNA extraction kits.

23. Perform RNA extraction from microdissected tissue using the PicoPure RNA Extraction kit or equivalent kit, according to the manufacturer's instructions.

RNA yields can be quantified using a small-volume spectrophotometer, such as a NanoDrop.

IN VITRO TRANSCRIPTIONAL AMPLIFICATION OF RNA

BASIC PROTOCOL 2

The following section describes the in vitro transcription (IVT) amplification of RNA from plant cells. It uses an oligo (dT)-T7 chimeric primer to preferentially select polyadenylated RNA species and then convert the RNA into antisense RNA through two rounds, each entailing sequential reverse transcription, conversion to double-stranded DNA using *E. coli* DNA polymerase I, and transcription using T7 RNA polymerase. Use RNase-free DEPC-treated water in all recipes and protocol steps.

NOTE: All centrifugation steps are performed in a benchtop microcentrifuge at room temperature.

Materials

T7-oligo(dT) primer (0.5 μg/μl):
(5′TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCG TTTTTTTTTTTTTTTTTTTT-3′)
RNA extracted from laser microdissection (LM) sample (see Basic Protocol 1)
Diethylpyrocarbonate (DEPC; Sigma)
dNTP mix (10 mM, Intermountain Scientific)
SuperScript II Reverse Transcriptase (200 U/μl, Invitrogen) containing:
5× first-strand buffer
0.1 M DTT
RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/μl, Invitrogen)
T4 gene 32 protein (5 μg/μl, USB)
E. coli DNA polymerase I (10 U/μl, New England Biolabs) containing:
10× DNA polymerase I buffer
β-Nicotinamide adenine dinucleotide hydrate (β-NAD^+; 260 μM, min. 98% from yeast, Sigma)
Ribonuclease H (RNase H; 2 U/μl, Invitrogen)
E. coli DNA ligase (10 U/μl, New England Biolabs)
T4 DNA polymerase (3 U/μl, New England Biolabs)
Phenol (Saturated, Fisher Scientific):
pH 6.6, BP1750I-400 (for step 10)
pH 4.3, BP1751I-400 (for step 18)
Chloroform (~0.75% ethanol as preservative, Technical grade, Fisher Scientific)
QIAquick PCR Purification Kit including:
Qiagen 250 columns
Buffer PB
Buffer PE
Bufffer EB
Sodium acetate (100 mM, pH 5.2, certified ACS, Fisher Scientific)
MEGAscript T7 Kit (Ambion) including:
rNTP solutions
10× reaction buffer
T7 RNA polymerase enzyme mix
RNase-free DNase I
Nuclease-free H_2O
RNeasy Mini Kit (50 columns; Qiagen) includes:
1.5- and 2.0-ml collection tubes
RNase-free reagents and buffers (including Buffer RLT and Buffer RPE)

Ethanol (Absolute, Aaper Alcohol)
Random hexamer primer (1 μg/μl, Roche Diagnostics)

Microcentrifuge tubes (nuclease-free)
Heating block or water bath preset to 16°C, 37°C, 42°C, 65°C, 70°C, 95°C
Concentrator/evaporator
Vortex

Perform first-round RNA amplification

1. For each reaction, mix the following components in a nuclease-free microcentrifuge tube:

0.5 μg/μl T7-oligo(dT) primer	1 μl
total RNA extracted from LM sample	20 to 100 ng
H_2O (DEPC-treated)	to 10.5 μl.

2. Incubate the samples 10 min at 65°C and cool on ice for 5 to 10 min.

   The primer anneals to poly(A)-containing RNA during this step, and thereby attaches a copy of the T7 RNA polymerase promoter sequence to the cDNA molecules that will be synthesized in the following steps

3. Collect the samples by "quick-spin" centrifugation 30 sec at 600 × *g*.

4. Add 8.5 μl of the following mixture to each tube:

10 mM dNTP mix	1 μl
5× first-strand buffer	4 μl
0.1 M DTT	2 μl
40 U/μl RNAseOUT	1 μl
5 μg/μl T4 gene 32 protein	0.5 μl.

   NOTE: *When preparing a cocktail master mixture to accommodate multiple samples, excess reagents should be prepared to compensate for reagent loss during pipetting.*

   RNaseOUT is an inhibitor of RNaseA, RNaseB, and RNaseC type ribonucleases, and is added to help prevent degradation of RNA samples during the ensuing reverse transcriptase reaction.

5. Mix gently and add 1 μl of Superscript II (200 U/μl) to each tube.

6. Incubate 1 hr at 42°C. If necessary, at this point the samples can be stored indefinitely at –20°C.

   Reverse-transcription of the RNA occurs during this step to form single-stranded DNA that contains the T7 RNA polymerase promoter at 5′ end.

7. Add 130 μl of the following mixture to each 20-μl reaction:

10× *E. coli* DNA polymerase I buffer	15 μl
10 mM dNTP mix	3 μl
260 μM β-NAD^+	15 μl
10 U/μl *E. coli* DNA polymerase I	4 μl
2 U/μl RNase H	1 μl
10 U/μl *E. coli* DNA ligase	1 μl
H_2O	91 μl.

   NOTE: *Prepare excess reagent mixture to compensate for reagent loss during pipetting.*

8. Mix gently and incubate 2 hr at 16°C.

   During this step, DNA polymerase I synthesizes second-strand DNA molecules while DNA ligase will ligate the new molecules into a single, uninterrupted DNA strand.

9. Add 2 μl of T4 DNA polymerase (3 U/μl) and incubate 10 min at 16°C.

   T4 DNA polymerase will fill in any remaining internal gaps in the second-strand DNA, and will fill in any leftover 5′ and 3′ overhangs to yield blunt ends.

10. Extract the double-stranded DNA with an equal amount of 1:1 phenol (pH 6.6)/chloroform (1:1).

11. Extract with an equal volume of chloroform and transfer the aqueous layer to a new microcentrifuge tube.

12. Purify the DNA using a Qiagen QIAquick PCR Purification column as follows:

    a. Add 35 μl of 100 mM sodium acetate (pH 5.2) to each tube.

    b. Add 500 μl of Buffer PB to each tube and mix by inverting.

    c. Proceed as per manufacturer's instructions until elution.

    d. Add 15 μl of H_2O to each column, allow the column to stand for 1 min, and centrifuge 1 min at maximum speed. Repeat once.

13. Concentrate the sample to 8 μl in a concentrator/evaporator at 50°C.

14. Prepare the reagents from the MEGAscript T7 Kit.

    a. Thaw the rNTP solutions, mix by vortexing, collect the sample by "quick-spin" centrifugation 30 sec at 600 × *g*, and place on ice.

    b. Thaw 10× reaction buffer, mix until the precipitate has dissolved, and keep at room temperature (not on ice).

15. Assemble the 20-μl reaction in the following order:

cDNA (end-product from step 13)	8 μl
rNTP mix (2 μl each of ATP, CTP, GTP, and UTP)	8 μl
10× reaction buffer	2 μl
T7 RNA polymerase enzyme mix	2 μl.

    NOTE: *When preparing a cocktail mixture to accommodate multiple samples, excess reagents should be prepared to compensate for reagent loss during pipetting.*

16. Incubate the reaction mix 5 hr at 37°C.

    During this step, T7 RNA polymerase will transcribe antisense RNA from the T7 RNA polymerase promoter sequence that was incorporated into the cDNA prepared above. This results in one round of RNA amplification.

17. Add 1 μl of RNase-free DNase I (2 U/μl) and incubate 15 min at 37°C.

    This step removes the cDNA template from the reaction mixture, leaving amplified RNA (aRNA) that is antisense in orientation.

18. Add 30 μl of nuclease-free H_2O to the sample and extract with an equal volume (50 μl) of 1:1 phenol (pH 4.3)/chloroform.

19. Extract with an equal volume of chloroform and transfer the aqueous layer to a new microcentrifuge tube.

    During steps 18 and 19, the newly synthesized aRNA is purified by extraction with organic solvents to denature and remove enzymes and other proteins.

20. Concentrate sample in RNeasy mini column:

    a. Add 350 μl of Buffer RLT (with 3.5 μl of 2-mercaptoethanol) and mix thoroughly by inverting.

b. Add 250 µl of absolute ethanol and mix thoroughly by pipetting. Do not centrifuge.

c. Apply the entire sample (700 µl) to an RNeasy minicolumn placed in a 2-ml collection tube.

d. Centrifuge 15 sec at 8000 × *g*, and discard the flowthrough.

e. Transfer the RNeasy column to a new 2-ml collection tube.

f. Pipet 500 µl of Buffer RPE onto the RNeasy column.

g. Centrifuge 15 sec at 8000 × *g*, and discard the flowthrough.

h. Add another 500 µl of Buffer RPE to the RNeasy column and centrifuge 2 min at 8000 × *g* to dry the RNeasy silica-gel membrane. Discard the flowthrough.

i. To elute, transfer the RNeasy column to a new 1.5-ml collection tube and pipet 15 µl of H_2O onto the RNeasy column.

j. Centrifuge 1 min at 8000 × *g*.

k. Pipet another 15 µl of H_2O onto the RNeasy column, and centrifuge 1 min at 8000 × *g*.

21. Concentrate the amplified RNA (aRNA) sample to 11 µl in a concentrator/evaporator at 50°C.

22. Use a 1-µl aliquot for RNA quantification.

    A low-volume spectrophotometer such as a NanoDrop is utilized.

Second-round RNA amplification

23. Assemble the first-strand reaction by mixing 1 µl of random hexamer primer (1 µg/µl) with 10 µl of aRNA and incubate 10 min at 70°C. Cool on ice for 5 min.

    This results in annealing of the random primers to the aRNA.

24. Collect the sample by "quick-spin" centrifugation 30 sec at 600 × *g*, and equilibrate the tube at room temperature for 10 min.

25. Add 8 µl of the following mixture to each tube:

10 mM dNTP mix	1 µl
5× first-strand buffer	4 µl
0.1 M DTT	2 µl
40 U/µl RNaseOut	0.5 µl
5 µg/µl T4 gene 32 protein	0.5 µl.

    NOTE: *When preparing a cocktail mixture to accommodate multiple samples, excess reagents should be prepared to compensate for reagent loss during pipetting.*

26. Mix gently, add 1 µl of Superscript II (200 U/µl), and incubate 1 hr at 37°C.

    This step generates first-strand cDNA from the aRNA.

27. Add 1 µl of RNase H (2 U/µl), and incubate 30 min at 37°C.

    This step removes the RNA strand from the RNA-DNA hybrids generated in the previous step.

28. Heat for 2 min at 95°C. Cool sample on ice for 5 min.

29. Add 1 µl of 0.5 µg/µl T7-oligo(dT) primer and incubate 5 min at 70°C. Cool the sample on ice for 5 min. Collect the sample by "quick-spin" centrifugation 30 sec at 600 × *g*.

    This step anneals the primer to the cDNA.

30. Incubate 10 min at 42°C, and place sample on ice for 5 min.

31. Add 128 μl of the following mixture to each tube:

10× *E. coli* DNA polymerase I buffer	15 μl
10 mM dNTP mix	3 μl
260 μM β-NAD^+	15 μl
10 U/μl *E. coli* DNA polymerase I	4 μl
2 U/μl RNase H	1 μl
H_2O	90 μl.

NOTE: *When preparing a cocktail mixture to accommodate multiple samples, excess reagents should be prepared to compensate for reagent loss during pipetting.*

32. Follow steps 8 to 20h of first-round RNA amplification.

33. To elute the aRNA, transfer the RNeasy column to a new 1.5-ml collection tube, pipet 30 μl of H_2O onto the RNeasy column, and centrifuge 1 min at 8000 × *g*.

34. Pipet another 30 μl of H_2O onto the RNeasy column, and centrifuge 1 min at 8000 ×*g*.

35. Use a 1-μl aliquot for RNA quantification; a low-volume spectrophotometer such as a NanoDrop can be used.

The purified, concentrated RNA may now be used in cDNA synthesis to generate templates for qRT-PCR, microarray analyses, or RNA-seq.

COMMENTARY

Background Information

The use of LM technology was first described for high-resolution analyses of gene expression in mammalian cells and tissues (Becker et al., 1996; Emmert-Buck et al., 1996; Luo et al., 1999), and has been especially utilized in analyses of the molecular pathogenesis of human disease (reviewed in Espina et al., 2007). Protocols utilizing LM to analyze gene expression in mammalian systems are presented in *UNIT 25A.1*. Owing to the relatively small amount of tissue harvested during a typical LM experiment, a key innovation in the use of LM for global expression profiling was the development of reliable protocols for the amplification of nucleic acids, including linear amplification of RNA using T7 RNA polymerase (Van Gelder et al., 1990; Eberwine et al., 1992). Indeed, the combined use of LM and RNA amplification has enabled analyses of gene expression from picogram quantities of RNA extracted from a single animal cell (Becker et al., 1996; Schütze and Lahr, 1998; Kamme et al., 2004). Further, the use of LM for proteomic analyses is hampered by the inability to amplify harvested proteins, such that its use is restricted to analyses of very abundant proteins extracted from a relatively large number of microdissected cells (Schad et al., 2005; Dembinsky et al., 2007; reviewed in Mustafa et al., 2008).

Initially, the biological and histological peculiarities of plant cells presented procedural challenges to the use of LM in plants. For example, many plant cells are especially small in comparison to animal cells, whereas other plant cells contain extremely large vacuoles harboring hydrolytic enzymes. Perhaps the greatest obstacle in adapting LM technology to plants is the presence of extremely rigid, interconnected, cellulosic cell walls that present a formidable barrier to laser cutting and cell harvesting. However, protocols are now developed for the fixation, infiltration, and embedding of a wide range of plant cell and tissue types amenable to laser microdissection-mediated transcriptional profiling, including the vasculature, leaf epidermis, hypocotyl, embryo, root, shoot apical meristem, organ excision zone, and fibers (Asano et al., 2002; Kerk et al., 2003; Nakazono et al., 2003; Casson et al., 2005; Klink et al., 2005; Schad et al., 2005; Woll et al., 2005; Jiang et al., 2006; Dembinsky et al., 2007; Ohtsu et al., 2007; Spencer et al., 2007; Wu et al., 2007; Yu et al., 2007; Zhang et al., 2007; Cai et al., 2008).

Critical Parameters and Troubleshooting

Tissue fixation

The fixative must penetrate plant tissues and arrest biological activities, while

preserving the cellular macromolecules (lipids, proteins, nucleic acids, carbohydrates) in a state that best approximates that found in living tissues and enabling their efficient extraction. Surveys of several chemical fixatives have concluded that reagents that coagulate or precipitate cellular molecules are superior to non-coagulative or cross-linking fixatives for use in LM-mediated RNA analyses (Nakazono et al., 2003; reviewed in Kehr et al., 2003; Day et al., 2005; Nelson et al., 2006). Thus, although non-coagulative fixatives such as formaldehyde and glutaraldehyde yield superior tissue histology, these are generally avoided for LM studies owing to the greatly reduced yields of RNA extracted from cross-linked tissues. In addition to the acetone fixative described in this unit, a number of different coagulating fixatives have been used for LM analyses of plants including ethanol, ethanol/acetic acid, and chloroform/acetic acid.

It is of critical importance that dissection times be minimized to 5 min or less per sample to avoid eliciting transcriptional responses to plant wounding. If longer dissection times are mandated, dissections should be performed while the plant tissues are immersed in fixative. In addition, fixation should be performed at cold temperatures to allow for the gradual penetration of plant tissues; rapid infiltration of chemical fixatives may shock plant tissues, introducing structural and anatomical artifacts due to cellular disruption.

Infiltration and embedding media

As described above for chemical fixation, infiltration of plant tissues must also be carried out gradually; sudden and drastic changes in chemical environments may generate extreme tissue anomalies (reviewed in Ruzin, 1999). Although this unit describes paraffin embedding, a number of plant LM studies have utilized fresh-frozen, cryo-embedded, and cryosectioned plant tissues (Asano et al., 2002; Nakazono et al., 2003; Casson et al., 2005; Schad et al., 2005). Several studies have reported improved yields of RNA extracted from cryo-sectioned tissues compared to paraffin-embedded samples (Goldsworthy et al., 1999; Gillespie et al., 2002); however, fast-freezing can cause vacuolar ruptures and wholesale anatomical disruptions that may prohibit the accurate microdissection of fine-scale plant tissue domains. Thus, despite the slight reduction in RNA yield, many researchers opt for the superior histological resolution obtained in paraffin-sectioned samples.

RNA extraction:Tissue-specific results

A number of commercially available RNA extraction kits are suitable for the isolation of minute concentrations of total RNA from microdissected plant cells, and are not individually evaluated here. However, RNA yields from LM-derived samples may vary considerably depending upon the specific plant tissue analyzed. When planning LM experiments, researchers must carefully consider the targeted tissue and empirically determine the appropriate number of cells to be microdissected. A single plant cell may contain <10 pg or as much as 100 pg of RNA, depending upon the tissue type (Zimmerman and Goldberg, 1977; Dixon et al., 2000). As a rule, fully differentiated plant cells may be quite large and extremely vacuolated, and contain far less RNA/μm^2 than the smaller, densely cytoplasmic cells found in undifferentiated and actively dividing tissues (reviewed in Nelson et al., 2006; authors' personal observations).

RNA amplification procedures

Typically, LM procedures are performed on <1000 plant cells, and yield between 5 and 100 ng of total RNA (reviewed in Day et al., 2005). Although these RNA yields are often sufficient to perform a few qRT-PCR reactions, most transcriptomics applications incorporate an RNA amplification procedure to generate several micrograms of amplified RNA (aRNA). In addition to the "in-house" RNA amplification protocol for T7 RNA polymerase based in vitro transcription (IVT) described in this unit, a variety of RNA amplification kits are commercially available and are not critically evaluated here. Similar to the IVT protocol described in this unit, many commercially available kits generate aRNA that is in antisense orientation, whereas other protocols produce sense-oriented aRNA. Before choosing a particular RNA amplification method, researchers planning to perform LM-microarray analyses should be particularly attentive to the strand orientation of the microarray probe elements to ensure that the aRNA is compatible with the array platform of choice.

Although capable of $>10^4$-fold amplification, IVT also introduces some degree of bias that may be exacerbated following multiple rounds of RNA amplification. Amplified RNA prepared in this manner is 3′ truncated and may contain some degree of non-linear amplification; some transcripts may amplify more efficiently than others, such that the relative transcript abundances may not be exactly equivalent to the starting mRNA. Notably,

these IVT-introduced biases tend to be systematic and reproducible such that direct comparisons of any two samples subjected to equivalent RNA amplification protocols are usually compensated for inherent biases (Nakazono et al., 2003; Schneider et al., 2004; Wilson et al., 2004; Day et al., 2007). Kerk et al. (2003) analyzed the linearity of IVT amplification using *Arabidopsis* RNA and reported correlation coefficients of 0.92 (unamplified RNA/amplified RNA) after a single round of RNA amplification versus 0.87 after two sequential amplifications, results that are in agreement with those reported by IVT kit manufacturers. Based on these data and similar results from separate studies (Luzzi et al., 2003), more than two rounds of IVT RNA amplification is usually discouraged. Although typical PCR can result in considerable differences in representation of different transcripts, as an alternative to IVT, PCR employing 12 cycles or fewer has also been employed to amplify nanogram quantities of LM-harvested RNA with less transcript truncation than IVT protocols and good correlation with quantitative analyses of unamplified RNA samples (Wilhelm et al., 2006; Day et al., 2007).

Anticipated Results

In their comparisons of RNA yield following LM of 1000 vascular or epidermal cells from maize seedlings, Nakazono et al. (2003) harvested 35 to 43 ng of total RNA corresponding to an average yield of 2 to 3 pg of RNA per individual cell. Following two rounds of amplification by IVT, these researchers generated from 24 μg to over 46 μg of aRNA, which translates to amplification rates of over 62,000-fold to more than 100,000-fold. Similar procedures performed on replicate samples of ten maize seedling SAMs, which contain significantly smaller cells than vascular or epidermal tissues, yielded average harvests of >10 μg of aRNA per mm^2 of microdissected SAM tissue (Zhang et al., 2007). Lastly, LM of ~700 parenchyma, collenchyma, or epidermis from cryosections of tomato fruit pericarp yielded from 5 ng to 50 ng of total RNA that, after two rounds of amplification, produced between 75 and 100 μg of aRNA (A. Arroyo and J. Rose, personal communication of unpublished data).

Laser microdissected plant RNAs amplified by IVT typically range from 0.2 kb to >2 kb (see Fig. 23A.5.1; Ohtsu et al., 2007) and are free of genomic contamination. Thus, the majority of aRNAs prepared following LM exhibit at least some degree of transcript truncation, the majority of which appears to be a by-product of the IVT amplification procedure rather than RNA shearing during laser-harvesting of plant tissue (authors' unpublished results).

Time Considerations

A single experienced individual can hand-dissect and fix at least twenty maize seedling shoots per hour. Following overnight fixation, sample infiltration and embedding takes 5 days. These steps may be shortened to as little as 3 days for less dense tissues such as *Arabidopsis* seedlings, whereas 8 to 10 days may be required to infiltrate and embed more compact tissues such as 20 day-after-pollination maize kernels. Once embedded in Paraplast and kept at 4°C, samples may be stored indefinitely. After experience is gained in microtome sectioning of maize shoot apices, four to five samples per hour can be readily processed. However, following microtome sectioning and fixation to slides, samples should be placed under vacuum desiccation at 4°C and used for LM as quickly as possible. The authors have not attempted to perform LM of RNA on slides that were prepared more than 14 days in advance. Several mm^2 of SAM tissue can be laser-microdissected in a single day, although the time required for LM can vary tremendously depending upon the abundance of targeted tissues the effort required to locate the specific cells/tissues of interest. Optimization of LM settings, which must be performed for every tissue type, typically requires <10 min. Finally, whereas the kit-based protocols for RNA extraction of LM tissues can be completed in <1 hr, IVT-based RNA amplification requires an investment of two full days.

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Internet Resources

http://www.palm-microlaser.com/dasat/index.php?cid=100113&conid=0&sid=dasat

Offers product information for PALM MicroLaser Systems at Carl Zeiss.

MOLECULAR METHODS FOR DISCOVERY OF DIFFERENTIALLY EXPRESSED GENES

Production of a Subtracted cDNA Library

PRODUCTION OF A SUBTRACTED LIBRARY

For some experiments, a complete cDNA library (*UNIT 5.8A*) is unnecessary and instead, a subtracted cDNA library is useful. A subtracted cDNA library contains cDNA clones corresponding to mRNAs present in one cell or tissue type and not present in a second type. This cDNA library is used to isolate a set of cDNA clones corresponding to a class of mRNAs, or to aid in the isolation of a cDNA clone corresponding to a particular mRNA where the screening procedure for the cDNA clone is laborious because a specific DNA or antibody probe is unavailable. A technique known as differential screening is an alternative to creating subtracted libraries (see Commentary).

In this protocol, the tissue, library, RNA, or cDNA designated with a [+] contains the target or desired sequence(s), and that which is to be subtracted from the [+] is termed [−]. Since relatively few recombinants are obtained after subtraction, this protocol is for a cDNA library constructed in the λgt10 vector or its equivalent, which allows a high cloning efficiency and permits elimination of nonrecombinants; however, the protocol can be used to produce subtracted cDNA libraries in any vector system.

[+] cDNA with *Eco*RI ends and [−] cDNA with blunt ends are prepared. The [−] cDNA is digested with *Rsa*I and *Alu*I to give small blunt-ended fragments. The [+] cDNA inserts are mixed with a 50-fold excess of fragmented [−] cDNA inserts, the DNAs in the mixture are heated to melt the double-stranded DNA, and the single-stranded insert DNA is allowed to hybridize. After hybridization, annealed cDNA inserts are ligated to λgt10 arms, packaged, and transfected.

The only [+] cDNA likely to regenerate double-stranded fragments with an *Eco*RI site at each end are those sequences for which no complementary fragments were present in the [−] cDNA. The subsequent cloning step allows the selection and amplification of these fragments.

Materials

[+] and [−] cDNA libraries (ATCC or Stratagene)
TE buffer (*APPENDIX 2*)
*Eco*RI and 10× EcoRI buffer (*UNIT 3.1*)
0.5 M EDTA, pH 8.0 (*APPENDIX 2*)
10% sucrose solution (*UNIT 5.3*)
1.5% and 2% agarose gels (*UNIT 2.5A*)
TBE buffer (*APPENDIX 2*)
95% and 70% ethanol
S1 nuclease (Sigma; *UNIT 3.12*) and 10× S1 nuclease buffer (*UNIT 3.4*)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1A*)
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
*Alu*I and 10× *Alu*I buffer (*UNIT 3.1*)
*Rsa*I (*UNIT 3.1*)
Deionized formamide (Fluka, IBI, or American Bioanalytical)
20× SSC (*APPENDIX 2*)
1 M $NaPO_4$, pH 7.0 (see recipe)

Contributed by Lloyd B. Klickstein
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10% sodium dodecyl sulfate (SDS)
10 mg/ml yeast tRNA
24:1 chloroform/isoamyl alcohol
Phosphatased λgt10 arms (Stratagene)
10× T4 DNA ligase buffer (*UNIT 3.4*)
T4 DNA ligase (measured in cohesive-end units; New England Biolabs; *UNIT 3.14*)
E. coli C600*hflA* (Table 1.4.5)
λ phage packaging extracts (Stratagene)
Suspension medium (SM; *UNIT 1.11*)

SW-28 rotor and 38-ml centrifuge tubes (Beckman) or equivalent
0.4-ml microcentrifuge tube

Additional reagents and equipment for construction of recombinant DNA libraries (*UNITS 5.5 & 5.6*), large-scale DNA preps from plasmids (*UNIT 1.7*) or phage (*UNIT 1.13*), sucrose gradients (*UNIT 5.3*), agarose gel electrophoresis (*UNIT 2.5A*), production and growth/maintenance of λ phage libraries (*UNITS 5.8, 25B.2,* and *1.9-1.13*), plating and titering libraries (*UNITS 6.1 & 6.2*), hybridization (*UNIT 6.3*), and radiolabeling probes (*UNIT 3.4*)

Prepare the insert DNA

1. Prepare or obtain cDNA libraries from the [+] and [−] cells or tissue sources.

   A major advantage of this protocol is that a subtracted library may be prepared from existing libraries, which is highly recommended. Complementary DNA libraries from many species and tissue sources are widely available and considerable time may be saved by obtaining preexisting [+] and [−] libraries to be used in this protocol.

   Alternatively, prepare ≥1 μg [+] cDNA with EcoRI ends and 10 μg [−] cDNA with blunt ends (stop the [−] cDNA synthesis before adding linkers) from poly(A)$^+$ [+] and poly(A)$^+$ [−] RNA, respectively (UNITS 5.5 & 5.6). If this is done, proceed to step 13.

   The protocol assumes that the [+] and [−] libraries are bacteriophage λ libraries. If the vector for either is a plasmid, only 100 μg of each is needed (scale down steps 2 and 3 by 1/10) and the inserts should be purified by agarose gel electrophoresis rather than by sucrose gradient centrifugation.

2. Perform large-scale (2 to 3 liters) DNA preps of both the [+] and [−] libraries to obtain >1 mg DNA from each library. Resuspend the DNA at 1 mg/ml in TE buffer.

Digest the DNA

3. Digest 1 mg of each library DNA in a 1.5-ml microcentrifuge tube as follow (final volume 1.167 ml):

   1 ml library DNA (1 mg)
   0.117 ml of 10× EcoRI buffer
   0.05 ml *Eco*RI (1000 U).

   Mix by shaking and incubate 5 hr at 37°C. Stop the reaction by adding 40 μl of 0.5 M EDTA, pH 8.0, and incubate 10 min at 65°C. During the digestion, prepare four 10% to 40% sucrose gradients in 38-ml SW-28 tubes (*UNIT 5.3*). Label two tubes [+] and two tubes [−].

   Internal EcoRI sites present in the cDNA inserts will be cut. If this occurs, the partial-length cDNA clone obtained through this procedure can be used to generate a probe with which to screen the initial [+] library for a full-length clone. The advent of newer vectors (e.g., λZAP; see Fig. 1.10.8) with cloning sites for enzymes such as NotI will nearly eliminate this difficulty.

Run the sucrose gradients

4. Mix each digest with an equal volume of 10% sucrose solution and carefully layer the digested [+] library DNA onto the two 10% to 40% sucrose gradients labeled [+]. Split the sample between the two tubes evenly. Similarly, load the [−] DNA onto the two gradients labeled [−]. Centrifuge the gradients overnight (18 to 24 hr) at 122,000 × *g* (26,000 rpm in an SW-28 rotor), 20°C.

   The insert fragments will remain near the top of the gradient while the phage arms will migrate half the length of the tube.

5. Harvest the gradients by gently removing 0.2-ml fractions from the top of the tube with a pipettor. Place each fraction into a separate, labeled microcentrifuge tube at 4°C.

   Twenty fractions per gradient are sufficient, as the insert DNA is small and barely enters the gradient under these conditions. Save the remainder of the gradient until the fractions containing the inserts have been identified, just in case!

Recover the DNA

6. Identify the tubes containing the insert DNA by analyzing 20 µl of every other fraction on a 1.5% agarose gel made in TBE buffer.

7. Precipitate the insert DNA: add 0.3 ml TE buffer and 1.0 ml of 95% ethanol to each tube, mix, and place at −20°C for 2 hr or on dry ice for 15 min.

   Because the sucrose gradient buffer contains 1 M NaCl, there is sufficient NaCl in the fractions for precipitation of the DNA. The sucrose in the gradient fractions must be diluted in order to successfully precipitate the DNA. A 2- to 3-fold dilution of these low-density fractions is adequate. A greater dilution of the higher density fractions is necessary in order to obtain high recoveries of DNA following ethanol precipitation.

8. Thaw and collect the DNA by microcentrifugation at high speed for 15 min. Aspirate the supernatant and save until DNA recovery has been checked. Add 0.5 ml of 70% ethanol to each tube. Recentrifuge, aspirate the ethanol supernatants, and dry the pellets.

9. Resuspend and pool the fractions containing insert DNA from the [+] library in TE such that the final concentration is 0.2 mg/ml. Store the DNA at −20°C.

10. Resuspend and pool the insert DNA from the [−] library in 100 µl TE buffer and place on ice. Save an aliquot of 400 ng of each [+] and [−] cDNA separately, to be used in evaluating the final library produced.

    Expect recoveries of >10 to 15 µg of insert DNA from 1 mg of total library DNA. The aliquots of [+] and [−] DNA, alternatively, may be radiolabeled and used as probes for differential screening of the [+] library (see Commentary).

Remove EcoRI ends from [−] DNA

11. Remove the *Eco*RI ends from the [−] DNA by mixing in the following order (final volume 112 µl):

    100 µl [−] insert DNA (10 to 15 µg)
    11 µl 10× S1 nuclease buffer
    1 µl 1:500 S1 nuclease (2 U).

    Mix by vortexing, briefly microcentrifuge, and incubate 30 min at 37°C.

12. Stop the reaction by adding:

    5 µl 0.5 M EDTA, pH 8.0
    200 µl TE buffer
    300 µl phenol/chloroform/isoamyl alcohol.

    Vortex. Microcentrifuge 1 min to separate the phases and transfer the upper, aqueous phase to a new tube. Add 30 µl of 3 M sodium acetate, pH 5.2, and 700 µl ethanol. Freeze, then collect the DNA by centrifugation as in steps 7 and 8. Resuspend the washed and dried pellet in 100 µl TE buffer.

Digest the [−] DNA with AluI and RsaI

13. Digest the S1 nuclease–treated [−] insert DNA to small fragments with *Alu*I and *Rsa*I by adding in the following order (final volume 121 µl):

    100 µl [−] insert DNA (10 to 15 µg)
    12 µl 10× AluI buffer
    5 µl *Alu*I (50 U)
    4 µl *Rsa*I (60 U).

    Mix by vortexing, briefly microcentrifuge, and incubate 3 hr at 37°C. Add 5 µl of 0.5 M EDTA, pH 8.0, and incubate 10 min at 65°C to stop the reaction. Remove and save 5 µl of the digest for evaluation by electrophoresis.

14. Add 200 µl TE buffer and 300 µl phenol/chloroform/isoamyl alcohol; extract and ethanol precipitate the DNA as in step 12. Resuspend the washed and dried pellet in TE buffer at 1.0 µg/µl.

15. Check the 5-µl aliquot from step 13 by running a 2% agarose minigel (in TBE buffer) and ethidium bromide–staining. The [−] DNA fragments should be between 50 and 200 bp in length.

Hybridize the DNA

16. Hybridize the [+] insert DNA with the [−] DNA fragments. Add in the following order to a 0.4-ml microcentrifuge tube (final volume 51 µl):

    25 µl deionized formamide (50% vol/vol final)
    10 µl [−] DNA fragments (10 µg)
    1 µl [+] insert DNA (0.2 µg)
    12.5 µl 20× SSC (5× final)
    0.5 µl 1 M $NaPO_4$, pH 7.0 (10 mM final)
    0.5 µl 0.1 M EDTA, pH 8.0 (1 mM final)
    0.5 µl 10% SDS (0.1% final)
    1.0 µl 10 mg/ml yeast tRNA (0.2 mg/ml final).

    Mix by vortexing, briefly microcentrifuge, and place tube in a bath of boiling water for 5 min. Briefly microcentrifuge again and incubate 18 to 24 hr at 37°C.

    The boiling step melts the DNA strands. During the hybridization step, only [+] sequences not present in the [−] DNA will find their complementary strands and regenerate clonable, double-stranded fragments with EcoRI ends. A [+] sequence also present in the [−] DNA will hybridize with at least one of the AluI/RsaI [−] fragments, forming a partially single-stranded, partially double-stranded molecule without clonable ends.

17. Add 200 µl TE buffer and transfer the mixture to a 1.5-ml microcentrifuge tube. Wash the hybridization tube with 250 µl TE buffer and add it to the hybridization mix (the volume is now 500 µl). Add 500 µl phenol/chloroform/isoamyl alcohol, vortex, and microcentrifuge 1 min to separate the phases.

18. Transfer the upper, aqueous phase to a new tube. Reextract this phase with 500 µl chloroform/isoamyl alcohol as in step 17. Recover the aqueous phase and add 50 µl of 3 M sodium acetate, pH 5.2, and 1 ml ethanol. Precipitate as in steps 7 and 8. Resuspend the washed and dried pellet in 12 µl TE buffer.

Chloroform/isoamyl alcohol extraction ensures removal of SDS and formamide.

Ligate the DNA

19. Ligate the insert DNA to λgt10 (not λgt11) phage arms by adding in the following order (final volume 25 µl):

12 µl insert DNA
10 µl λgt10 phosphatased phage arms (10 µg)
2.5 µl 10× ligase buffer
0.5 µl T4 DNA ligase (200 U).

Mix by gently pipetting up and down and incubate overnight at 12° to 15°C.

Package and plate the library

20. Start a fresh overnight culture of *E. coli* C600*hflA* and the next morning, package the ligation from step 19 with 8 to 10 commercial λ phage packaging extracts according to manufacturer's instructions.

The vector λgt10 is used here because it permits selection of recombinants when grown on the appropriate host. Ten micrograms of bacteriophage λ vector is roughly an equimolar amount of EcoRI ends with respect to the input [+] DNA, the ends of which must be considered even though only a small fraction of the [+] insert DNA is clonable after the melting and hybridization steps. The recommended 10 µg of vector and no less than 8 to 10 packaging extracts will ensure a library of good complexity.

21. Add suspension medium (SM) to the packaging mixtures and pool them in a 5-ml polypropylene tube to a final volume of 2 ml. Add two drops of chloroform, shake by hand for 3 sec, and allow the chloroform to settle.

22. Plate 0.2 ml packaged phage with 3 ml fresh C600*hflA* plating bacteria on each of ten 150-mm plates as described in the library amplification protocol (*UNIT 25B.2*); however, allow the plates to incubate overnight at 37°C.

23. The following morning, count the number of plaques on a representative plate and multiply by 10 to determine the total number of recombinants in the library.

Typically, 300 to 15,000 phage per library are obtained.

24. Elute the plates with SM as in *UNIT 25B.2* or directly select individual plaques for screening.

Evaluate the library

25. Evaluate a newly prepared subtracted library as described in *UNIT 5.8A* (first support protocol).

The best approach is to amplify the library and differentially screen duplicate nitrocellulose filters from a single 150-mm plate of 20,000 to 40,000 recombinants. Hybridize one lift with a total [+] cDNA probe and the other with a total [−] cDNA probe. The total [+] and [−] cDNA probes are prepared by radiolabeling some of the [+] and [−] cDNA saved from step 10. Most clones should hybridize with the [+] probe and few with the [−] probe. Evaluation by screening the library with a probe for proteins such as actin or tubulin would not be appropriate, since the expected result is no hybridization (or only a few), which may occur for a variety of reasons.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

1 M $NaPO_4$, pH 7.0

A: 1 M Na_2HPO_4
B: 1 M NaH_2PO_4
Add B to A until pH = 7.0

COMMENTARY

Background Information

The practical consequence of creating a subtraction library is considerable enrichment of the target cDNA clones. For example, a subtracted cDNA library was used to isolate T cell antigen receptor cDNAs. By hybridizing T cell cDNA to B cell mRNA, and selecting the single-stranded cDNA molecules by hydroxylapatite column chromatography, the T cell antigen receptor cDNAs were significantly enriched. The cDNA was then hybridized back with the T cell mRNA from which it was derived and the double-stranded RNA-DNA hybrids were selected, carried through second-strand cDNA synthesis, and the resulting cDNA was cloned (Hedrick et al., 1984). Thus, a large percentage of the clones in the subtracted library were T cell–specific. All clones in the subtracted library would have been present in a library constructed from the T cell line without the subtraction step; the objective was to obtain a library so enriched that clones derived from it could be screened by random selection.

Two major disadvantages to the approach outlined above are that poly(A)$^+$ RNA from both [+] and [–] source is required and the hybridizations, hydroxylapatite columns, and library production with a very small amount of cDNA are technically difficult. A conceptually different approach, termed deletion enrichment, has been undertaken in the construction of a genomic library enriched for Y chromosome–specific sequences (Lamar and Palmer, 1984). In this case, [+] DNA (male) and an excess of [–] DNA (female) fragments were mixed, denatured, hybridized, and [+] DNA that did not hybridize to [–] was selected by a cloning step. Production and selection of a library were accomplished simultaneously.

The subtracted cDNA library protocol described here is an adaptation of the deletion enrichment method to cDNA libraries and is recommended over the other because (1) it is conceptually and practically simple, involving only standard laboratory techniques; (2) it does not involve the handling of RNA, which can be problematic; (3) it may be performed with DNA prepared from already existing libraries, eliminating the potential time and expense involved in the preparation of fresh tissue or cells; and (4) with slight modifications, either cDNA or genomic subtracted libraries may be prepared.

A disadvantage of this approach is that if no [+] or [–] cDNA libraries are available, they must first be made or cDNA must be synthesized, requiring an extra few days to a week. A second disadvantage of this or any other subtraction protocol is that clones containing reiterated sequences—e.g., an *Alu* repeat in the 3′ untranslated region—would be eliminated from the library on that basis. Thus, the representation of a clone containing a reiterated sequence would be lower than expected, with only partial-length cDNAs present after subtraction.

A good alternative to creating a subtracted library is differential screening of a library known to contain the target clone(s). In one well-characterized experiment, duplicate lifts from a lymphoid tissue cDNA library were screened with total B cell cDNA and total T cell cDNA probes. B cell–specific clones were identified as plaques that hybridized with a B cell cDNA probe and not with a T cell probe (Tedder et al., 1988). A potential drawback to the differential screening approach is that rare sequences will have very low specific probe concentrations in the mixture and thus might not hybridize to the DNA from a target plaque in a reasonable period of time (e.g., overnight).

Critical Parameters

Because the *Eco*RI ends of [+] insert cDNA must remain intact through the sucrose gradient and hybridization steps, the nuclease inhibitor and bacteriostatic agent EDTA is present in both steps. In contrast, S1 nuclease digestion destroys the *Eco*RI ends of [–] cDNA, ensuring that all clones in the final library are derived from [+] cDNA. Restriction digestion of [–] DNA with *Alu*I and *Rsa*I increases the molar ratio of [–] to [+] DNA while not increasing the

mass of [–] cDNA, which can inhibit subsequent steps. The boiling step prior to hybridization is essential to melt the cDNA to its single-stranded form. The hybridization conditions favor the annealing of fragments >50 bp and a relatively long hybridization is required to permit reannealing of rare [+] cDNA. Hybridization buffer must be diluted at least 10-fold in order to successfully phenol extract, chloroform extract, and ethanol precipitate the DNA. A full 10 µg of phage vector arms must be added in the ligation reaction. This represents only an equimolar amount of *Eco*RI ends with respect to the number of ends from the [+] cDNA, and is by no means an excess.

A major advantage of this protocol is that it may be performed with reagents, enzymes, and supplies routinely available in a typical molecular biology laboratory. Sucrose for gradients should be molecular biology grade (DNase-free). λgt10 phosphatased arms and packaging extracts should be obtained commercially unless large volume use is anticipated, in which case "homemade" arms and extracts would be more economical.

Troubleshooting

The initial *Eco*RI digestion, the sucrose gradients, and the *Alu*I and *Rsa*I digestion of [–] DNA are monitored by minigel electrophoresis. If difficulties such as incomplete digestion or poor separation occur here, see the commentaries of *UNITS 3.1 & 5.3*. Poor recovery of DNA is usually not a problem, since at least 10 µg of DNA is present at each precipitation. Once beyond these steps, there is no method for evaluation short of determining the titer and composition of the subtracted library. Possible adverse outcomes include too few clones, too many clones, or no enrichment for [+] clones (see Anticipated Results).

When too few clones are obtained, check the λgt10 phage arms, packaging extracts, and host bacteria by cloning a test insert. If $>1 \times 10^7$ PFU/µg test insert are obtained, the problem may be that the *Eco*RI ends of the [+] cDNA have been destroyed or there is an inhibitor of one of the later steps present in the DNA. Evaluate these possibilities by cloning [+] DNA after the sucrose step and measuring the efficiency, and by cloning a test insert with and without post-hybridization DNA added to the test insert ligation.

If too many clones are obtained, the problem is usually contamination of one or more reagents with phage, non–*E. coli* C600*hflA* bacterial host, or failure to denature the [+] or [–] DNA prior to hybridization. Too many clones may also be obtained if the S1 nuclease digestion of [–] DNA did not work, which can be evaluated by cloning some of the [–] DNA directly. Alternatively, check the S1 nuclease step by digesting some M13 DNA under the same conditions and monitoring the reaction by agarose gel electrophoresis.

If the subtraction did not work, duplicate filters screened with [+] and [–] total cDNA probes as described in step 20 will have roughly equal numbers of positive clones. The most likely explanation is that the S1 nuclease digestion was incomplete. Check the S1 step by cloning 100 ng of the [–] DNA; $<10^3$ PFU/µg insert is expected.

Anticipated Results

The number of clones obtained depends on the similarity of sources of [+] and [–] cDNA. For a subtracted cDNA library prepared from B cell [+] and T cell [–] cDNA, 5500 recombinants were obtained, 15% of which were immunoglobulin clones. Because of the high level of similarity between B cells and T cells, this result probably represents a minimum number to be expected. Investigators using this protocol have reported 300 to 15,000 phage per library. Twenty to fifty percent of the clones in a well-constructed library will be [+]-specific; most of the remainder will be abundant cDNAs present in both [+] and [–] cDNA. Five to ten percent of clones *not* [+]-specific may have inserts that are not released by *Eco*RI and probably represent aberrant ligation of [–] fragments into the vector.

Time Considerations

Once [+] and [–] total library DNA is obtained, perform the *Eco*RI digestion during the day and run the sucrose gradient overnight. On the second day harvest the gradients, perform the S1 digestion of the [–] cDNA, and store the precipitated DNA overnight. On the third day, digest the [–] DNA with *Alu*I and *Rsa*I and set up the hybridization overnight. The fourth day, ligate the cDNA to the λgt10 vector overnight and start an overnight culture of host cells. Package and plate over the following night. The protocol may be interrupted at any ethanol precipitation overnight or over the weekend.

Literature Cited

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Tedder, T.F., Strueli, M., Schlossman, S.F., and Saito, H. 1988. Isolation and structure of a cDNA encoding the B1 (CD20) cell-surface antigen of human B lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 85:208-212.

Contributed by Lloyd B. Klickstein
Brigham and Women's Hospital
Boston, Massachusetts

PCR-Based Subtractive cDNA Cloning

Subtractive cloning is a powerful technique that allows isolation and cloning of mRNAs differentially expressed in two cell populations. In the generalized subtraction scheme illustrated in Figure 25B.2.1, the cell types to be compared are the [+] or tracer cells and the [−] or driver cells, where mRNAs expressed in the tracer and not the driver are isolated. Briefly, tracer nucleic acid (cDNA or mRNA) from one cell population is allowed to hybridize with an excess of complementary driver nucleic acid from a second cell population to ensure that a high percentage of the tracer forms hybrids. Hybrids that form include sequences common to both cell populations. Hybrids between the tracer and driver, and all driver sequences, are removed in the subtraction step. The unhybridized fraction is enriched for sequences that are preferentially expressed in the tracer cell population.

The method described here (see Basic Protocol) uses double-stranded cDNA (ds cDNA) as both tracer and driver and is modified from protocols devised by Sive and St. John (1988) and Wang and Brown (1991; see Background Information and Fig. 25B.2.2). Reciprocal subtractions are performed between two cell populations, A and B: that is, genes preferentially expressed in A more than in B are isolated, as are genes expressed

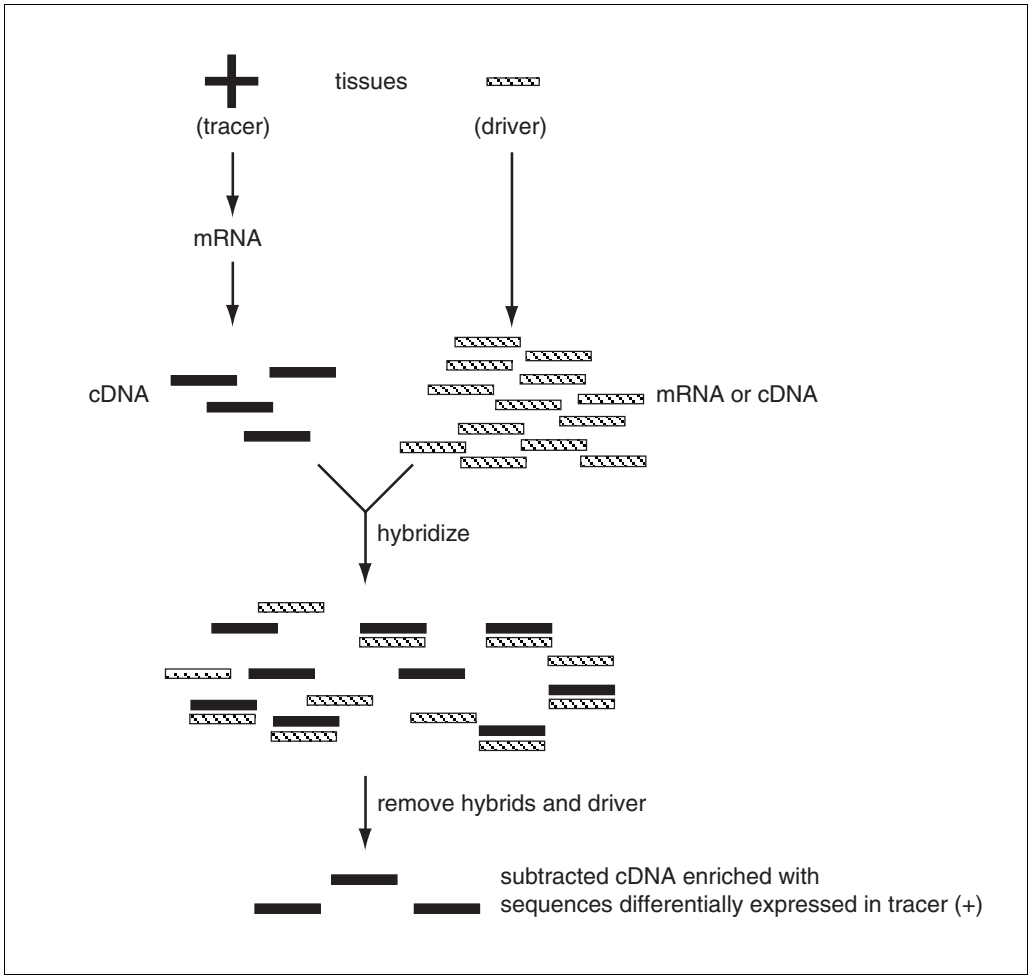

Figure 25B.2.1 Generalized subtraction scheme. Tracer cDNA from the + cell population is hybridized to >10-fold excess driver mRNA or cDNA from the – cell population. The resulting hybrids and excess driver are removed to enrich for cell type–specific sequences in the tracer. The subtraction may be repeated for further enrichment.

Contributed by Mukesh Patel and Hazel Sive

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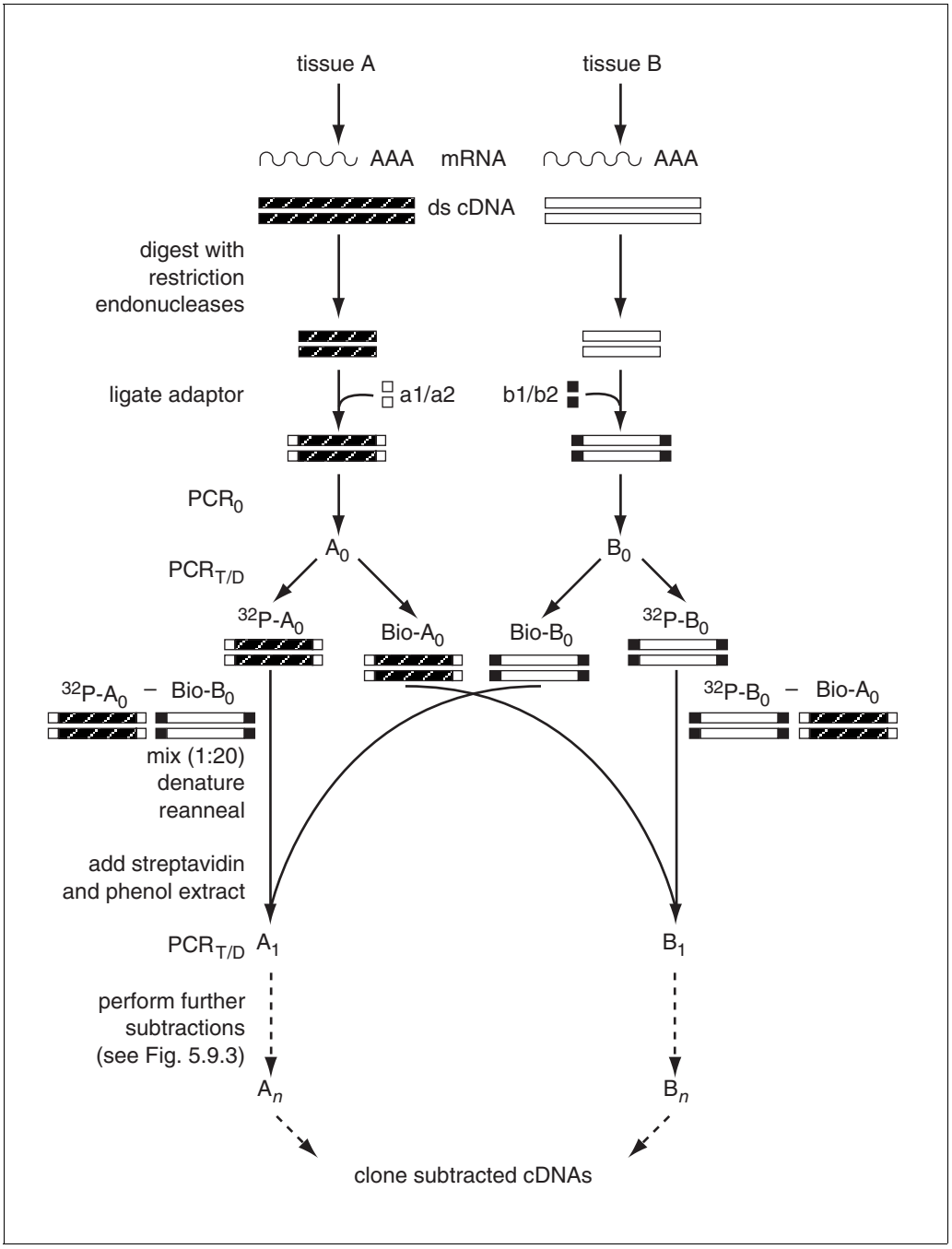

Figure 25B.2.2 Basic steps in PCR-based cDNA subtraction cloning. mRNAs purified from tissues A and B are used to synthesize double-stranded cDNA by standard methods. The resulting cDNAs are then digested with restriction endonucleases that have 4-bp recognition sequences. Two different sets of adapters (a1/a2 and b1/b2) are ligated to the two sets of digested cDNA. The cDNAs are amplified with the appropriate primers (a1 or b1) to yield A_0 and B_0. Two sets of subtractions are performed ($A_0 - B_0$ and $B_0 - A_0$). In each case the tracer is labeled with small amounts of [α^{32}P]dCTP, and the driver is labeled with bio-11-dUTP during PCR synthesis. Tracer and driver cDNAs are mixed at a ratio of 1:20, denatured, and allowed to reanneal. Driver/driver and tracer/driver hybrids are removed by treatment with streptavidin and extraction with phenol. This results in an enrichment of sequences found at greater abundance in tracer versus driver to yield A_1 and B_1. Further subtractions are performed after another round of amplification using the appropriate cDNAs (see Fig. 5.9.3). When the subtractions are completed, the cDNAs are cloned into an appropriate vector for analysis.

preferentially in B more than in A. The method uses the polymerase chain reaction (PCR) to amplify cDNAs after each subtraction to prepare tracer and driver for the next subtraction. This makes it possible to begin with very small quantities of cells and, by performing repeated subtractions, achieve maximal enrichment of differentially expressed genes in both cell populations. The progress of subtraction is monitored by slot blot hybridization (see Support Protocol). Differentially expressed cDNA sequences are used to construct a subtracted cDNA library.

STRATEGIC CONSIDERATIONS

For this method ds cDNA, full-length (if possible) and prepared from cell types A and B using oligo(dT) as first-strand primer, is the starting material. The ds cDNA is digested by restriction endonucleases to obtain short cDNA fragments. This prevents preferential PCR amplification of naturally small cDNAs. Next, each of the two cDNA samples is ligated to different adapters and amplified by PCR to obtain a large amount of material.

In the first (and subsequent) PCR amplification step, both tracer and driver cDNAs are made for each cell type to allow subtractions in both directions. The first subtractions are A_0 tracer – B_0 driver and B_0 tracer – A_0 driver. Tracer cDNA is made partially radioactive so the success of subtractions can be monitored. Driver cDNA is biotinylated during PCR by incorporating bio-11-dUTP to provide a basis for separation of hybrids and driver. Tracer and driver are mixed, denatured, and allowed to reanneal at a driver cDNA/tracer cDNA ratio of 20:1 and a driver concentration of ≥2 mg/ml (or for a driver with fragment sizes of 200 bp, 15 μmol/liter). In order to achieve this concentration, hybridizations are performed in small volumes (5 to 10 μl). Subtractions are performed in driver excess to ensure that the reannealing rate is a function of the driver concentration only and to drive hybridization of tracer as close to completion as possible. Subtractions are performed either for a short period of time to remove sequences that are common to both A and B and abundant in both, or for much longer to remove rarer sequences that are common to both A and B (see Critical Parameters).

After annealing, tracer/driver and driver/driver hybrids are efficiently removed by addition of streptavidin (a protein that specifically and tightly binds biotin) and extraction with phenol. Biotinylated nucleic acid that has bound streptavidin is taken into the organic phase or remains at the interface (Sive and St. John, 1988). Unhybridized tracer or tracer hybrids are not removed by the streptavidin/phenol treatment because they are not biotinylated and so remain in the aqueous phase. This constitutes subtraction and enrichment for differentially expressed genes. cDNAs remaining after the first set of subtractions are termed A_1 and B_1; these are used for the next round of subtraction.

The subtraction sequence is shown in Figure 25B.2.3. The number of subtractions necessary depends primarily on the complexity of the cDNAs, where complexity refers to the total number of different cDNAs, or fragments of cDNA, from each cell type (Davidson, 1986). The complexity should not be confused with the number of differentially expressed cDNAs, which is only a subset of the total cDNA populations. The greater the complexity of the starting mRNA pool (or, in general, the greater the number of cell types contributing to the starting mRNA), the more subtractions will be required. Ideally, subtraction should be repeated until no more cDNA is removed after hybridization and/or until the subtracted cDNAs (A_n and B_n) do not cross-hybridize. In practice, with the scheme described here, this is usually between five and twenty subtractions.

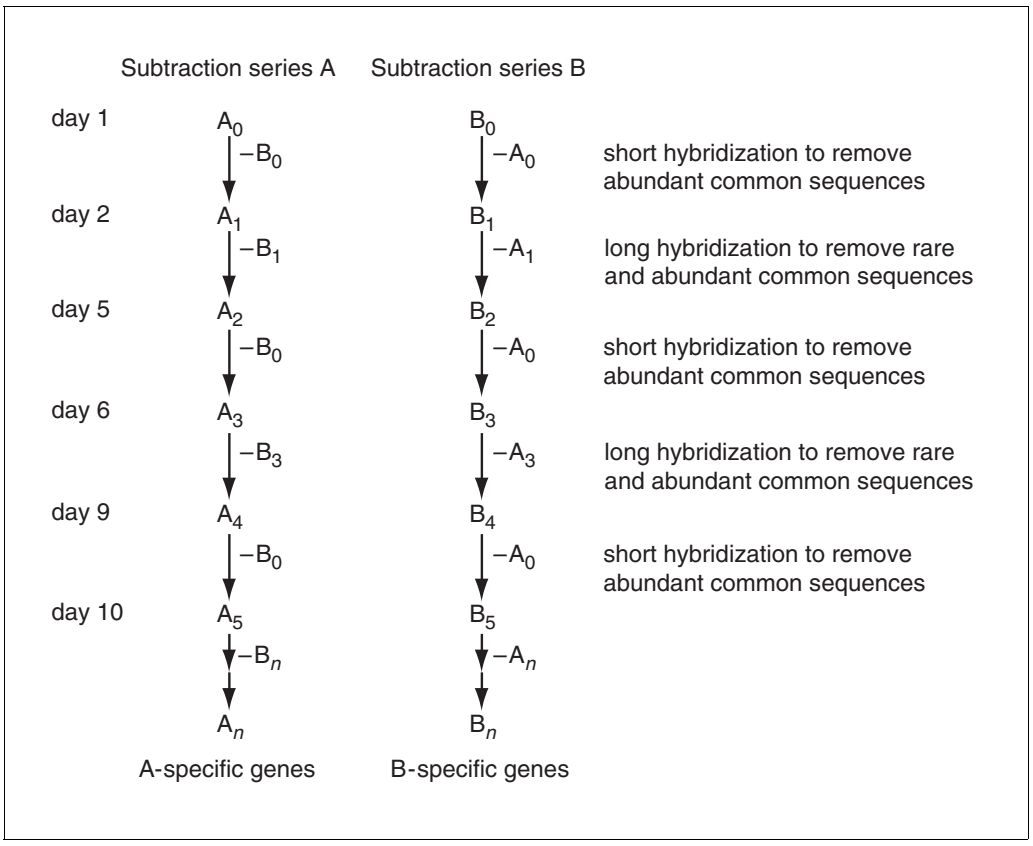

Figure 25B.2.3 Sequence of subtractions. The order of subtractions performed is outlined here for the first five subtractions. The approximate timescale and the hybridization length for each subtraction is indicated along with the primary purpose for each subtraction. Subtractions alternate between a short (2-hr) subtraction with A_0 or B_0 as driver and a long subtraction (30- to 40-hr) with A_n or B_n as driver. A_0 and B_0 are not normalized, that is, they contain an excess of abundant mRNAs or cDNAs and are therefore used to ensure that abundant common sequences are removed. Conversely, $A_1 - A_n$ and $B_1 - B_n$ are enriched for rarer sequences and therefore remove rare common sequences more efficiently than does A_0 or B_0. The progress of the subtractions is monitored by slot blot hybridization after every three to four subtractions. When the degree of enrichment is satisfactory (>20-fold differential; that is, when A_n hybridizes to itself better than to B_n >20-fold), then the subtracted cDNAs (A_n and B_n) are cloned into appropriate vectors for clonal analysis.

BASIC PROTOCOL

CONSTRUCTION OF SUBTRACTED cDNA LIBRARIES

This protocol describes preparation of libraries of subtracted cDNA clones that represent differentially expressed genes prepared from two cell populations. Each cDNA is ligated to a specific adapter and then the two sets of cDNAs are amplified by PCR to provide large amounts of starting material. Part of the starting material is radiolabeled to provide tracer cDNA to monitor subtraction efficiency; part is biotinylated to provide driver cDNA to facilitate removal of hybrids after annealing. Tracer cDNA from cell population A is hybridized to driver cDNA from population B and vice versa. Tracer/driver and driver/driver hybrids are removed by exposure to streptavidin and phenol extraction, leaving subtracted tracer cDNAs enriched for differentially expressed genes for each population. The sequences are enriched further by repeated rounds of amplification and hybridization. The progress of subtraction is monitored by slot blot hybridization (see Support Protocol). Finally, the subtracted cDNAs are ligated into vectors and used to create libraries that can be screened for individual differentially expressed genes.

Materials

Double-stranded cDNA (ds cDNA) for cell types A and B (*UNIT 5.5*)
*Alu*I and 10× *Alu*I buffer (see recipe)
*Rsa*I
10, 15, and 75 mM ATP
10 U/µl T4 polynucleotide kinase and 10× T4 polynucleotide kinase buffer (see recipe)
Oligonucleotide primers
  3 µg/µl a1: 5′-TAG TCC GAA TTC AAG CAA GAG CAC A-3′
  2.5 µg/µl a2: 5′-CTC TTG CTT GAA TTC GGA CTA-3′
  3 µg/µl b1: 5′-ATG CTG GAT ATC TTG GTA CTC TTC A-3′
  2.5 µg/µl b2: 5′-GAG TAC CAA GAT ATC CAG CAT-3′
10 U/µl T4 DNA ligase and 10× T4 DNA ligase buffer (see recipe)
40% (w/v) polyethylene glycol 8000 (PEG 8000)
25:24 (v/v) phenol/chloroform (made with buffered phenol; *UNIT 2.1*)
Chloroform
5 U/µl *Taq* DNA polymerase and 10× *Taq* DNA polymerase buffer (see recipe)
25 mM $MgCl_2$
10 mM 4dNTP mix (*UNIT 3.4*)
Mineral oil, PCR-grade, sterile
800 Ci/mmol [α^{32}P]dCTP (10 Ci/µl)
Driver dNTP mix (see recipe)
Ethanol
1 and 5 M NaCl
HEPES buffer (see recipe)
2× hybridization buffer for subtractions (see recipe)
Streptavidin solution (see recipe)
*Eco*RI and 10× *Eco*RI buffer (see recipe) or *Eco*RV and 10× *Eco*RV buffer (see recipe)
pBluescript vector cut with *Eco*RI
pBluescript vector cut with *Eco*RV
Tranformation-competent bacterial strain (*UNIT 1.8*)
Radiolabeled subtraction probes (see Support Protocol)

0.5-ml PCR tubes
Sephacryl S-300 spin columns (Pharmacia Biotech)
Beckman Accuspin FR centrifuge with swinging-bucket rotor or equivalent
Thermal cycler
Anion-exchange PCR spin columns (Qiagen)
1.5-ml microcentrifuge tubes, silanized (*APPENDIX 3B*)
Hand-held Geiger counter
Heating block

Additional reagents and equipment for restriction endonuclease digestion (*UNIT 3.1*), agarose gel electrophoresis (*UNIT 2.5A*), chromatography to remove oligonucleotide fragments (*UNIT 2.6*), phenol/chloroform extraction and ethanol precipitation (*UNIT 2.1A*), anion-exchange (Qiagen) column purification of oligonucleotides (*UNIT 2.1B*), spectrophotometric quantitation of nucleic acids (*APPENDIX 3D*), hybridization of slot blots (*UNIT 2.9B & 2.10*; also see Support Protocol), bacterial transformation (*UNIT 1.8*), plating libraries (*UNIT 6.1*), preparing replica filters (*UNIT 6.2*), hybridizing replica filters (*UNIT 6.3*), preparing minipreps of plasmid DNA (*UNIT 1.6*), and sequencing plasmid DNA (*UNIT 7.4A & 7.4B*)

Digest ds cDNA with restriction endonucleases

Double-stranded cDNA (ds cDNA) is digested with frequent-cutting restriction endonucleases into 200- to 600-bp fragments so PCR will not be biased towards smaller fragments.

1. For each set of ds cDNA (A and B) set up two digestions (*Alu*I and *Alu*I + *Rsa*I) as follows:

   30 ng ds cDNA
   3 µl 10× *Alu*I buffer
   10 U *Alu*I *or* 10 U *Alu*I + 10 U *Rsa*I
   H_2O to 30.0 µl.

   Incubate overnight at 37°C to ensure complete digestion.

   Any other frequent-cutting restriction endonucleases may be used, but enzymes that generate blunt ends are preferable. If an enzyme does not generate blunt-ended DNA fragments, an additional filling-in or chewing-back step is required.

   This protocol starts with double-stranded cDNA, full length if possible and primed with oligo(dT), from each cell type being compared (see UNIT 5.5). Commercially available cDNA-synthesis kits from several companies (e.g., Pharmacia Biotech or Life Technologies) work well, even with ≤100 ng poly(A)$^+$ RNA. Silanized tubes and glycogen are used during ethanol precipitation to avoid loss of cDNA. Sephacryl S-400 columns (Pharmacia Biotech) can be used to purify the synthesized cDNA, which must be cuttable and clean enough for adapter ligation. Between 10 and 100 ng cDNA is a suitable quantity for this digestion.

2. Heat inactivate restriction endonucleases by incubating the reactions ≥10 min at 65°C.

   Some restriction endonucleases are not susceptible to heat inactivation; phenol/chloroform extraction (UNIT 2.1A) is required to remove them.

Prepare adapters

The adapters are made by annealing kinased oligonucleotide primers a1 or b1 to unphosphorylated primers a2 or b2, respectively.

3. Kinase oligonucleotides a1 and b1 using the following reaction (25 µl per reaction):

   18.0 µl H_2O
   2.5 µl 10 mM ATP
   2.5 µl 10× T4 polynucleotide kinase buffer
   1.5 µl 3 µg/µl oligonucleotide a1 *or* oligonucleotide b1
   0.5 µl 10 U/µl T4 polynucleotide kinase.

   Incubate 60 min at 37°C.

   It is important that the ligated adapters do not contain or regenerate the restriction endonuclease recognition site in case the enzymes are not totally inactivated (see Critical Parameters).

4. Heat inactivate the kinase by incubating 20 min at 65°C.

5. Add 1.5 µl of 2.5 µg/µl oligonucleotide a2 *or* 2.5 µg/µl oligonucleotide b2 to form a1/a2 or b1/b2 adapters. Mix and microcentrifuge briefly at maximum speed. Incubate 10 min at 45°C.

   The adapters can be stored at –20°C at this stage.

Ligate adapters to cDNA

Adapters are ligated onto the cDNAs and excess adapters are removed.

6. Set up ligation reactions in 0.5-ml PCR tubes for each set of cDNAs using the appropriate adapter (130 µl per reaction):

   63 µl H_2O
   13 µl 10× T4 DNA ligase buffer
   30 µl 40% PEG 8000
   1 µl 15 mM ATP
   10 µl *Alu*I-digested cDNA
   10 µl *Alu*I/*Rsa*I-digested cDNA
   2 µl a1/a2 adapter *or* 2 µl b1/b2 adapter
   1 µl 10 U/µl T4 DNA ligase.

   Mix and incubate 2 hr at 16°C.

7. Incubate reactions >10 min on ice.

8. Prepare Sephacryl S-300 spin columns according to manufacturer's instructions.

9. Add 1 µl of 75 mM ATP and 1 µl T4 polynucleotide kinase to each ligation reaction. Incubate 30 min at 37°C.

10. Extract the ligation reaction with 1 vol of 25:24 phenol/chloroform, then with 1 vol chloroform.

11. Centrifuge the reaction mixture through a prepared Sephacryl S-300 spin column—i.e., 2 min at 400 × *g* in a Beckman Accuspin FR with a swinging-bucket rotor, room temperature—to remove unligated adapters.

    Approximately 130 µl ligated cDNA will come through the column.

    Ligated cDNAs may also be separated from unligated adapters by agarose gel electrophoresis (UNIT 2.5A) followed by electroelution (UNIT 2.6).

Amplify ligated cDNA

Ligated cDNA is amplified by PCR to obtain large amounts of cDNA (A_0, B_0).

12. Set up a PCR mixture for each of the two sets of cDNAs (50 µl per reaction):

    35 µl H_2O
    5 µl 10× *Taq* DNA polymerase buffer
    3 µl 25 mM $MgCl_2$
    1 µl 10 mM 4dNTP mix
    0.5 µl 2.5 µg/µl oligonucleotide a2 *or* oligonucleotide b2
    5 µl 0.2 ng/µl ligated A cDNA *or* B cDNA
    0.5 µl 5 U/µl *Taq* DNA polymerase.

    Add a few drops of sterile PCR-grade mineral oil to cover the reaction.

13. Amplify the cDNA using the following PCR program:

30 cycles:	1 min	94°C (denaturation)
	1 min	50°C (annealing)
	2 min	72°C (extension)
	25 sec	72°C (autoextension)

    If available, use the autoextension function of the thermal cycler (e.g., Perkin-Elmer 480). Alternatively, for thermal cyclers without autoextension, increase the extension time from 2 to 4 min.

    This amplification should yield ~10 µg A_0 and B_0 cDNAs.

    The reaction product can be stored overnight at 4°C or longer at −80°C.

14. Analyze 5 to 10 µl of the amplified cDNAs by agarose gel electrophoresis (*UNIT 2.5A*) to determine the size ranges of amplified cDNAs.

The size of amplified cDNAs should be between 150 bp and 1.5 kb with most ~250 bp.

Prepare labeled tracer and driver DNAs

Radioactive tracer DNA is required for monitoring subtraction efficiency; biotinylated driver DNA is required for removing hybrids by streptavidin binding and phenol extraction.

15. For both sets of amplified cDNAs, set up the following tracer synthesis PCR (100 µl per reaction):

77 µl H_2O
10 µl 10× *Taq* DNA polymerase buffer
6 µl 25 mM $MgCl_2$
2 µl 10 mM 4dNTP mix
1 µl diluted [^{32}P]dCTP
1 µl 2.5 µg/µl oligonucleotide a2 *or* b2
2 µl A_0 *or* B_0 cDNA (~0.4 µg)
1 µl 5 U/µl *Taq* DNA polymerase.

Add a few drops of sterile PCR-grade mineral oil to cover the reaction.

The amount of cDNA used for these initial A_0 and B_0 tracer synthesis reactions is 400 ng; this may be decreased but use ≥40 ng for the first amplification. In subsequent amplifications, use 5 to 10 ng A_n or B_n cDNA.

These reactions yield ^{32}P-labeled tracer cDNA ([^{32}P]A_0 and [^{32}P]B_0 in the first round and [^{32}P]A_n and [^{32}P]B_n in subsequent rounds; see Fig. 25B.2.2).

16. For both sets of amplified cDNAs, set up three or four driver synthesis PCRs (100 µl per reaction):

73.3 µl H_2O
10 µl 10× *Taq* DNA polymerase buffer
6 µl 25 mM $MgCl_2$
6.7 µl driver dNTP mix
1 µl 2.5 µg/µl oligonucleotide a2 *or* b2
2 µl A_0 *or* B_0 cDNA (1 to 5 ng)
1 µl 5 U/µl *Taq* DNA polymerase.

Add a few drops of sterile PCR-grade mineral oil to cover the reaction.

The driver dNTP mix contains 0.5 mM bio-11-dUTP and 1.0 mM dTTP. In the authors' hands this ratio of bio-11-dUTP/dTTP gives the highest overall subtraction efficiency and still allows efficient base pairing.

These reactions yield biotinylated driver cDNA (Bio-A_0 and Bio-B_0 in the first round and Bio-A_n and Bio-B_n in subsequent rounds; see Fig. 25B.2.2).

17. Use the PCR amplification program described in step 13 for tracer and driver synthesis.

18. Purify amplified cDNAs away from unincorporated nucleotides, primer, and salts using a commercial anion-exchange PCR spin column (Qiagen) as directed by the manufacturer (*UNIT 2.1B*).

An alternate way to purify the PCR products is by agarose gel purification (UNIT 2.5A), but care must be taken to avoid contamination with other DNAs.

19. Determine the yields by spectrophotometric quantitation of nucleic acids (*APPENDIX 3D*).

    Typical amplifications yield 12 to 16 µg ^{32}P-labeled cDNA per 100-µl tracer reaction and 7 to 10 µg biotinylated cDNA per 100-µl driver reaction.

    The quality and size range of the purified cDNA should be checked using agarose gel electrophoresis (UNIT 2.5A) after every third PCR amplification before proceeding to the next subtraction step. The size range should not change significantly.

Anneal tracer and driver

This is a hybridization between ^{32}P-labeled tracer and biotinylated driver cDNAs.

20. Set up two hybridization reactions ([^{32}P]A_n – Bio-B_n and [^{32}P]B_n – Bio-A_n). Ethanol precipitate 1 µg radiolabeled tracer and 20 µg biotinylated driver DNAs in a 1.5-ml silanized microcentrifuge tube without freezing. Air dry the pellet and when just dry, resuspend in 5 µl HEPES buffer by gentle pipetting. Monitor resuspension of the pellet with a hand-held Geiger counter.

    A small radioactive pellet should be clearly visible. By not freezing during ethanol precipitation, the possibility of a large salt pellet is avoided.

    Resuspension of the pellet sometimes requires a little patience; warming the tube to 60°C usually helps. Also check that none of the counts (i.e., cDNA) are stuck to the pipet tip, as this can greatly reduce the subtraction efficiency. The use of silanized pipet tips may help reduce sticking.

    The pellet should not be resuspended in a larger volume because this will lower the concentration of driver, and hence the reannealing rate.

21. Transfer resuspended DNA to a 0.5-ml PCR tube. Add 5 µl of 68°C 2× hybridization buffer for subtractions. Mix by gentle pipetting and add a few drops of sterile PCR-grade mineral oil to cover the DNA solution. Microcentrifuge briefly at maximum speed.

    If a pellet is visible, the DNA has come out of solution.

22. Incubate the two tubes 10 min at 95°C and cool slowly over 1 hr to 68°C. Continue incubation 2 hr at 68°C (short hybridization).

    Either a thermal cycler or a heat block may be used for this step.

    Subsequent hybridizations alternate between long (30- to 40-hr) hybridizations during which both rare and abundant common sequences form hybrids, and short (2-hr) hybridizations during which only abundant common sequences form hybrids.

Remove biotinylated annealed and single-stranded DNA

Tracer/driver and driver/driver hybrids and biotinylated single-stranded driver cDNA are removed by addition of streptavidin and extraction with phenol/chloroform.

23. Mix 7 µl of 1 M NaCl with 140 µl HEPES buffer and warm to 68°C. Add to the hybridization reaction to dilute the reaction. Mix and microcentrifuge briefly at maximum speed. Cool to room temperature.

24. Remove 5 µl from each tube and save (total pre-phenol extraction counts).

25. Add 15 µl streptavidin to each tube. Vortex and incubate 5 min at room temperature.

26. Extract each tube with an equal volume 25:24 phenol/chloroform. Retain the aqueous phases and transfer to new tubes.

27. Add 10 µl streptavidin to each tube containing aqueous phase. Mix and incubate 5 min at room temperature.

28. Extract twice with phenol/chloroform and twice with chloroform. Measure the volume of the aqueous layer for each tube.

    The volume for each reaction should be ~150 µl.

    The aqueous phase contains A_n and B_n cDNA.

29. Remove 5 µl of the aqueous layer from each tube (total post-phenol extraction counts).

    Use either scintillation or Cerenkov counts of the pre- and post-phenol extraction samples to determine efficiency of subtraction. The percent tracer cDNA removed is calculated by the following equation:

    $$\% \text{ tracer removed} = 100 - (\text{total post-phenol counts} \times 100/\text{total pre-phenol counts})$$

    The subtracted material can be stored at −20°C.

Perform further subtractions

Further rounds of subtraction are performed using subtracted cDNAs from the previous round as template for PCR synthesis of tracer and driver cDNAs. Additional rounds of subtraction, with alternating short and long hybridization steps, continue enriching for the differentially expressed genes.

30. Repeat the subtractions (steps 15 to 29) using A_n or B_n tracer cDNA and the appropriate driver cDNA as determined by the subtraction strategy (see Fig. 25B.2.3). Use A_0 or B_0 drivers for short (2-hr) hybridizations and A_n or B_n drivers for long (30- to 40-hr) hybridizations. Monitor the progress of subtraction by slot blot hybridization (see Support Protocol).

    Between five and twenty rounds of subtraction are usually sufficient to isolate cDNAs for differentially expressed genes.

Clone subtracted cDNAs

Subtracted cDNAs are ligated into a vector and cloned to permit screening of individual clones.

31. Amplify 5 µl of the subtracted cDNAs (A_n and B_n) using the program described in step 13. Purify PCR products with a commercial anion-exchange PCR spin column (e.g., Qiagen; *UNIT 2.1B*).

32. Digest the cDNAs with the appropriate restriction endonucleases that cut within the adapters (e.g., *Eco*RI and *Eco*RV for the adapters used here).

    Taq DNA polymerase may survive phenol/chloroform extraction, so it may help to purify the cDNAs by treating the amplified reaction with proteinase K, extracting with phenol/chloroform, and precipitating with ethanol before digestion.

    Digestion may be omitted if blunt-ended ligations are to be performed. PCR amplification often results in the addition of an extra adenosine at the 3′ end; this should be removed by Klenow treatment (UNIT 3.16) if blunt-ended ligations are to be performed. Alternatively, the subtracted cDNAs may be cloned into a T-vector (UNIT 15.4).

33. Purify digested cDNAs by phenol/chloroform extraction and ethanol precipitation.

34. Ligate the DNA into an appropriate vector (*UNIT 3.16*; e.g., pBluescript digested with *Eco*RI or *Eco*RV).

    Any convenient vector may be used (see Critical Parameters and Troubleshooting). Using a vector with blue-white selection is useful because it allows immediate assessment of the proportion of the library that contains inserts.

35. Transform the vector into a transformation-competent bacterial strain (*UNIT 1.8*).

If the subtractions were done to (or nearly to) completion and most of the colonies contain inserts, then it should be possible to pick colonies at random and check for differential expression. Alternatively, use the following steps to assess the quality of the library.

Assess subtracted libraries

Replica filters of the library are probed to assess for percent differentially expressed clones and to provide an indication of the success of the subtractions.

36. Plate out the subtracted library (*UNIT 6.1*).

It is worth titrating the library first (UNIT 1.3) to obtain individual colonies. It is also important to determine the percentage of colonies that have inserts and the sizes of the inserts (UNIT 5.8). The insert size should be ~250 bp. If the insert size is >500 bp, consider the possibility that the inserts may be double inserts.

37. Prepare four replica lifts from each primary filter (*UNIT 6.2*).

38. Denature, neutralize, and cross-link the lifts according to the manufacturer's instructions (also see *UNIT 6.2*).

39. Use subtracted probes (see Support Protocol, step 5) to hybridize the replica filters.

Comparison of filters probed with A_n versus B_n identifies those clones that are probably differentially expressed in the starting A_0 and B_0 cDNAs and also indicates what proportion of the library contains differentially expressed genes. Further rounds of subtraction may be desirable if only a small number of the clones seem to be differentially expressed. The filter probed with a common abundant gene should give very few or no positive signals if the subtractions were done to completion. Finally, probing with a known differentially expressed gene(s) gives another indication of how well the subtractions have worked. If the library evaluation suggests that no further subtractions are needed, analyze individual clones in the library.

Sort through the library

The number of differentially represented clones from the subtracted library is assessed by sequencing and/or gridding.

40. Pick 50 to 100 differentially expressed clones from the library either randomly (if the library assessment indicates most of the clones are differentially expressed) or based on a differential hybridization screen using A_n and B_n as probes. Prepare a miniprep of plasmid DNA (*UNIT 1.6*).

41. Sequence the inserts in each of the plasmid DNAs (*UNIT 7.4A & 7.4B*) and group together clones containing the same sequences.

DNA sequence analysis software such as that from DNAStar is helpful.

If most of the clones analyzed initially are the same, they should be subtracted out to reveal rarer transcripts. This is done by pooling the identified clones and using them to make driver that is then used for subtraction with A_n or B_n tracer. Alternatively, the library can be plated out and the lifts probed with mixed probe from the sequenced clones (≤20 sequences/mixed probe). Clones that do not hybridize have not yet been sequenced and should be analyzed. If all the clones seem to be differentially expressed but a few are particularly prevalent, then another way to reveal rare transcripts is to normalize A_n and B_n (or self-subtract them—i.e., $A_n - A_n$ and $B_n - B_n$) for a short period of time. These procedures greatly reduce the work involved in sorting through the library.

42. Determine whether the clones are truly differentially expressed in the starting tissues by RNA expression analysis—e.g., northern blot hybridization (*UNIT 4.9*), RNase protection assay (*UNIT 4.7*), quantitative RT-PCR (*UNIT 15.5*), or in situ hybridization (*UNITS 14.3 & 14.7*).

SUPPORT PROTOCOL

SLOT BLOT HYBRIDIZATION TO MONITOR SUBTRACTION

After every three to four subtractions, the progress of enrichment for differentially expressed genes is monitored by slot blot hybridization (also see *UNITS 2.9B & 2.10*).

Additional Materials *(also see Basic Protocol)*

cDNA from each subtraction (see Basic Protocol, step 28)
3 M NaOH
2 M ammonium acetate, pH 7.0
Probe dNTP mix (see recipe)
Sephadex G50/80 spin column (Pharmacia Biotech) in sterile 1-ml syringe

Additional reagents and equipment for slot blotting (*UNIT 2.9B*) and hybridization (*UNIT 2.10*)

1. Denature 1200 ng cDNA from each subtraction ($A_n - B_n$ and $B_n - A_n$) by adding 0.1 vol of 3 M NaOH to cDNA and heating 30 to 60 min at 65°C.
2. Neutralize the DNA by adding 1 vol of 2 M ammonium acetate, pH 7.0.
3. Spot duplicate 100-ng aliquots of denatured and neutralized cDNA from each subtraction onto each of six or more slot blots (*UNIT 2.9B*).
4. Use cDNA from A_n, B_n, A_0, B_0, a gene expressed at high levels in both A and B, and one or more genes expressed differentially by A or B (or a gene used to spike the reaction) to prepare radiolabeled subtraction probes. Prepare a PCR mixture for each probe (50 µl per reaction):

   17.5 µl H_2O
   5 µl 10× *Taq* DNA polymerase buffer
   3 µl 25 mM $MgCl_2$
   2 µl probe dNTP mix
   20 µl [α-^{32}P]dCTP
   1 µl 2.5 µg/µl primer a2, primer b2, primer specific for gene expressed in both A and B, *or* primer specific for gene expressed differentially in A or B
   0.5 µl 4 ng/µl subtracted A_n or B_n cDNA or appropriate gene template DNA
   1 µl 5 U/µl *Taq* DNA polymerase.

   Add a few drops of sterile PCR-grade mineral oil to cover the reaction.

5. Amplify and label the probe using the following PCR program:

30 cycles	1 min	94°C (denaturation)
	1 min	50°C (annealing
	2 min	72°C (extension)

   This reaction yields a double-stranded probe; the probes should be denatured before hybridization.

6. Purify the probe by centrifuging it through a 1-ml Sephadex G50/80 spin column, 2 min at 170 × *g*, in a Beckman Accuspin FR with a swinging-bucket rotor, room temperature.

   Expect ~50 µl eluate after centrifugation.

7. Measure incorporation by counting a 1-µl fraction of the eluate in a scintillation counter.

   Routinely, incorporation is ~10^6 cpm/µl eluate.

8. Hybridize each slot blot with one of the above probes (*UNIT 2.10*).
9. Wash the blots to high stringency (*UNIT 2.10*).
10. Expose filters to X-ray film or a phosphoimaging plate (*APPENDIX 3A*).

    The A_n and B_n hybridizations are the most important because they reveal the degree to which A_n and B_n cDNAs still cross-hybridize with B_n and A_n cDNAs, respectively. In general, further subtractions are desired if the differential is <20-fold (that is, A_n hybridizes <20-fold better to itself than to B_n and vice versa). Probing the subtracted cDNAs with a highly expressed gene or with a differentially expressed gene gives another indication of how well the subtractions are advancing. Common abundant genes should become less abundant with increasing rounds of subtraction, and the known differentially expressed gene should become enriched in one series of cDNAs and depleted in the other. The A_0 and B_0 probes usually represent the common abundant genes and therefore behave accordingly; that is, they hybridize more strongly to cDNA from earlier rounds of subtraction and less so to later rounds. When the evaluation suggests that no more subtractions are required, then the A_n and B_n cDNAs should be cloned into an appropriate vector.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

AluI buffer, 10×

100 mM Bis-Tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane)·Cl, pH 7.0
100 mM $MgCl_2$
10 mM dithiothreitol (DTT; *APPENDIX 2*)
Store up to 6 months at −20°C

Driver dNTP mix

1.5 mM each dATP, dCTP, and dGTP
1.0 mM dTTP
0.5 mM bio-11-dUTP (Enzo Diagnostics)
Store up to 3 month at −20°C

EcoRI buffer, 10×

1 M Tris·Cl, pH 7.5 (*APPENDIX 2*)
500 mM NaCl (*APPENDIX 2*)
100 mM $MgCl_2$ (*APPENDIX 2*)
0.25% (v/v) Triton X-100
Store at −20°C

EcoRV buffer, 10×

100 mM Tris·Cl, pH 7.9 (*APPENDIX 2*)
500 mM NaCl (*APPENDIX 2*)
100 mM $MgCl_2$ (*APPENDIX 2*)
10 mM dithiothreitol (DTT; *APPENDIX 2*)
Store up to 6 months at −20°C

HEPES buffer

100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), pH 7.3
1 mM EDTA (*APPENDIX 2*)
Store at −20°C

Hybridization buffer for subtractions, 2×

50 mM HEPES, pH 7.3
10 mM EDTA (*APPENDIX 2*)
0.2% (w/v) SDS
1.5 M NaCl (*APPENDIX 2*)
Store up to 3 months at −20°C
To avoid cloudiness, add NaCl last and warm to 68°C

Probe dNTP mix

0.5 mM each dATP, dGTP, and dTTP
0.1 mM dCTP
Store up to 3 months at −20°C

Streptavidin solution

2 µg/µl streptavidin
0.15 M NaCl (*APPENDIX 2*)
HEPES buffer (see recipe)
Store up to 6 months at −20°C

T4 DNA ligase buffer, 10×

500 mM Tris·Cl, pH 7.8 (*APPENDIX 2*)
100 mM $MgCl_2$ (*APPENDIX 2*)
100 mM dithiothreitol (DTT; *APPENDIX 2*)
10 mM ATP
250 µg/ml BSA
Store up to 6 months at −20°C

T4 polynucleotide kinase buffer, 10×

700 mM Tris·Cl, pH 7.6 (*APPENDIX 2*)
100 mM $MgCl_2$ (*APPENDIX 2*)
50 mM dithiothreitol (DTT; *APPENDIX 2*)
Store up to 6 months at −20°C

Taq DNA polymerase buffer, 10×

100 mM Tris·Cl, pH 9.0 (*APPENDIX 2*)
500 mM KCl (*APPENDIX 2*)
1% (v/v) Triton X-1000
Store at −20°C

COMMENTARY

Background Information

Early subtractive cloning involved one or two rounds of hybridization using cDNA as tracer and mRNA as driver. cDNA/mRNA hybrids were removed by binding to hydroxylapatite columns maintained at 68°C. This scheme has two major limitations that prevented subtractive cloning from becoming a routine and frequently used technique. The first was that hydroxylapatite columns are cumbersome, making it difficult to separate single-stranded sequences from the hybrids. This problem has been largely overcome through the use of biotinylated driver sequences in combination with streptavidin treatment and phenol extractions (Sive and St. John, 1988; Sive et al., 1989), or streptavidin-conjugated magnetic beads (Uhlen, 1989; Straus and Ausubel, 1990).

A second problem with the original technique was the rapid decrease in the amount of cDNA present, making it very difficult to per-

form multiple rounds of subtraction or to clone the minute amounts of cDNA left after subtraction. Several different approaches have been used to tackle this second problem. One solution has been to construct directional phagemid libraries that can be converted into a single-stranded library; after the subtractions are performed, the remaining single-stranded plasmids are transformed into bacteria for amplification (Duguid et al., 1988; Rubenstein et al., 1990). The subtracted library can be used in further rounds of subtractions; however, the method is laborious and care must be taken to avoid contamination with the double-stranded (ds) forms of the phagemid. Contaminating ds phagemid DNA will not be subtracted away and will transform bacteria much more efficiently than single-stranded DNAs do, thus reducing the overall subtraction effect. Other methods have used cDNA attached to oligo(dT)-Latex in combination with the polymerase chain reaction (PCR). This allows the driver to be reused (Hara et al., 1993).

An alternative solution described in this protocol regenerates the cDNAs by PCR (Duguid and Dinauer, 1989; Wang and Brown, 1991). A problem with PCR is that it amplifies smaller fragments better than larger fragments and therefore selects for smaller mRNAs. Wang and Brown (1991) overcame this difficulty by cutting the original cDNAs to smaller sizes before PCR. This approach allows multiple rounds of subtractions and has allowed isolation of many genes that are differentially expressed in metamorphosis-stage *Xenopus* embryos after thyroid hormone treatment (Buckbinder and Brown, 1992). With the modified protocol detailed here, the authors have isolated many genes that delineate the early events of neural induction and anteroposterior patterning in *Xenopus* (Patel et al., unpub. observ.). The method described here is very sensitive and can isolate genes that are as little as 2- to 3-fold differentially expressed.

In the scheme described here, two cDNA populations are cross-subtracted—that is, A tracer is subtracted with B driver and B tracer with A driver. This allows isolation of genes expressed preferentially in A and genes expressed preferentially in B. Cross-subtraction has two other effects. The first is to increase the concentration of rare sequences relative to the concentration of abundant common sequences in the driver, because the latter rapidly hybridize (at low C_0t) and are removed by subtraction. This is termed normalization, as it normalizes or equalizes the concentrations of what were initially rare and abundant common cDNAs. In practice, it is not possible to reach a truly equalized representation of sequences, but the starting concentrations of different cDNAs can vary 10,000-fold, and after normalization this can be reduced to ~10-fold (Patanjali et al., 1991; Soares et al., 1994). Normalizing the driver makes it much more efficient at removing rare common sequences than an unnormalized driver. Normalizing the driver is essential when starting with tissues that have high mRNA complexity. It is, of course, also important that some of the subtractions be performed with a driver that still contains high levels of abundant common sequences (that is, the starting cDNA population, A_0 or B_0); otherwise these abundant sequences will never be removed.

Normalization could also be achieved by subtracting the driver against itself (self-subtraction). The reason cross-subtractions are used instead is that they provide a second benefit. One of the problems with any efficient subtraction scheme is that it may remove sequences expressed only a few-fold higher in one cell population than the other, and therefore allow isolation of only those sequences that are not expressed at all in the driver. Sequences expressed with ≤10-fold differential may be of great interest and can be isolated by cross-subtractions. Suppose that sequence G is present at a ratio of 1:5 in A_0/B_0, the starting cDNAs. If B_0 is subtracted with A_0, and vice versa, G will be removed somewhat from the resulting B_1; however, after the reciprocal $A_0 - B_0$ subtraction, relatively more G will be removed from the resulting A_1 than it was from B_1 because the driver (B_0) had a higher concentration of G than A_0 did. Thus, the ratio of G in A_1/B_1 will decrease, perhaps to 1:10. This enhanced relative difference in the level of G between A_1 and B_1 will be enhanced even more in subsequent cross-subtractions, to ultimately allow isolation of G as a differentially expressed clone. One problem here is that cross-subtracting can result in false positives (genes that are differentially represented in the final A_n and B_n cDNA populations, but not in the starting A_0 and B_0 cDNAs). This is a particular problem if the efficiences of the two subtraction series (A – B versus B – A) are different, but it can easily be checked after subtraction by asking whether a clone is differentially represented in the A_0 and B_0 starting cDNAs.

This protocol includes two modifications to the Wang and Brown (1991) method that the authors feel improve it. First, bio-11-dUTP is

incorporated into the driver as a means of biotinylating (Patel and Sive, unpub. observ.) in place of the photobiotinylation originally described (Sive and St. John, 1988) for two reasons. Incorporation of biotin during PCR amplification is extremely simple and does not require additional photobiotinylation steps. Substituting 30% of the dTTP with bio-11-dUTP in the amplification of driver nucleic acid gives maximal subtraction efficiency. With lower substitution, subtraction efficiency decreases, presumably because the density of biotin is not great enough; with greater substitution, subtraction efficiency also decreases, presumably because the biotin intereferes with base-pairing (Patel and Sive, unpub. observ.). Photobiotinylated nucleic acid is rather insoluble in aqueous solutions due to a long hydrocarbon linker arm; photobiotinylated driver sometimes precipitates out of the hybridization mix. Nucleic acids with biotinylated nucleotides incorporated during PCR seem as soluble as unmodified nucleic acids and precipitation in the hybridization mix does not occur, at least in in the authors' hands. Another method for incorporating biotinylated nucleotides is to use biotinylated primers for PCR (Rosenberg et al., 1994). Second, this protocol uses different adapters on the driver and tracer cDNAs. The original protocol (Wang and Brown, 1991) used the same adapters for both tracer and driver to ensure that all sequences in tracer and driver amplified to the same extent; however, this also meant an increased risk of driver carry-over into the next round of subtraction; such carried-over driver would be amplified along with the subtracted cDNA and would decrease subtraction efficiency. Using the different primers given here, the authors have observed essentially equivalent PCR efficiency for the two cDNA pools.

Several other methods have been used to isolate genes that are differentially expressed between two or more cell populations (see Table 25B.2.1)—random sampling (in which clones are randomly selected from a cDNA library), differential hybridization (in which probes made from the mRNAs of the two tissues being compared are used to screen a cDNA library, and clones that hybridize to one probe but not to the other are isolated), and differential display (*UNIT 25B.3*; in which partially random primers are used to amplify a subset of mRNAs expressed in a given cell type; these are then separated on an acrylamide gel and the bands between different samples compared). Of all the procedures, subtractive cloning is probably the most sensitive, and it is the method of choice to isolate as complete a set of differentially expressed genes as possible. The other methods allow isolation of a small number of differentially expressed genes and may be sufficient to obtain useful markers. Random sampling of a cDNA library is useful only if the two tissues to be compared contain a widely different spectrum of mRNAs.

Table 25B.2.1 A Comparison of Differential Screening Methods

Method	Advantages	Disadvantages
Subtractive cloning	Targets rare mRNAs (<0.001%) Targets complete set of differentially expressed RNAs Requires little starting material Nonisogenic tissues can be compared	Can generally only compare two tissues at one time Procedure can be long
Differential display	Requires little starting material Can compare more than two tissues or treatments at one time Procedure is relatively short	Targets only a subset of the differentially expressed genes Generally targets medium-abundant mRNAs Can yield many false positives
Differential hybridization	Procedure is relatively easy	Targets relatively abundant mRNAs (~0.1%)
Random sampling	Simple procedure; only cDNA libraries are required	Only useful for comparing very different tissues

Critical Parameters and Troubleshooting

Some of the more common problems that arise with this procedure and their solutions are listed in Table 25B.2.2.

RNA preparation

It is essential to start with a clean preparation of RNA that is free of any salts or other substances that may inhibit reverse transcription. The RNA should not be contaminated with even trace amounts of genomic DNA. In fact, all RNA preparations should be treated with RNase-free DNase, then checked for contaminating genomic DNA by PCR using primers for specific genes. Contaminating DNA alters the representation of the various mRNAs during the subtractions to create false positives.

Purity of oligonucleotides

The quality of the primers is crucial to the success of the procedure. It is worth purifying

Table 25B.2.2 Troubleshooting Guide for Subtractive cDNA Cloning

Problem	Probable cause(s)	Remedy
No amplified cDNAs visible on agarose gel	Failure of adapters to ligate to cDNA due to:	
	Kinasing of wrong primer	Kinase correct primer
	Inactive ligation buffers and/or enzymes	Test and replace as necessary
	Inhibitors in cDNA	Repurify cDNA by phenol/chloroform extraction and ethanol precipitation using glycogen as carrier
	Failure of PCR amplification due to inactive amplification buffer and/or enzymes	Test and replace as necessary
Median size range of amplified cDNAs >500 bp in A_0 and/or B_0	Incomplete digestion of cDNA before amplification due to:	
	Inhibitors in ds cDNA	Purify ds cDNA by phenol/chloroform extraction and ethanol precipitation using glycogen as carrier
	Inactive restriction buffers or enzyme	Test and replace as necessary
Low subtraction efficiency	Loss of DNA during ethanol precipitation or resuspension	Repeat with careful monitoring using hand-held Geiger counter
	Incomplete resuspension of DNA before hybridization	Avoid complete drying of DNA pellet before resuspension
		Warm sample to 60°C to aid resuspension
No or few colonies after cloning into vector	Incomplete digestion of DNA	Repurify DNA; treat with proteinase K, phenol/chloroform extract, ethanol precipitate, and wash with 70% ethanol
	Inactive enzymes or buffers	Test and replace as necessary
	Poor ligation efficiency	Repurify DNA; test and replace enzymes or buffers
	Low transformation efficiency	Test and replace competent cells
No or few differentially expressed genes	Contamination of RNA or cDNA with (genomic) DNA before ligation of adaptors	Restart with fresh RNA and treat it with DNase before reverse transcription

the primers to ensure they are full length and free of any salts or other inhibitors. The synthesized primers can be gel purified, although the authors prefer to use Nensorb Prep columns (Du Pont NEN). To use these columns, the 5′-trityl group on the primer must not be removed (see *UNIT 2.11* for more information about the synthesis of oligonucleotides).

Primer design

The two different adapters used during this protocol are created by annealing a 21-mer and a 25-mer oligonucleotide. The sequences of four primers that these authors have used successfully are listed in the Basic Protocol. These primers contain sites for *Eco*RI or *Eco*RV; however, different restriction endonuclease sites or other special features for particular vectors may be desirable, so this section outlines some considerations in primer design.

First, restriction endonucleases generally require at least four bases next to their recognition sequences to work efficiently. Second, primers should contain minimal secondary structure to maximize annealing to the target sequence. Third, there should be no similarity between the primers that make up one set of adapters and those that make up the second set. This is extremely important for the success of the subtractions, and it is essential to check for any cross-annealing by testing whether a primer from one set of adapters can amplify cDNA (or a test DNA fragment) ligated to the other adapter. Fourth, in order to perform the PCR amplifications for the two sets of cDNAs (A and B) at the same time, it is important that the primers have similar melting temperatures (T_m). Fifth, the T_m should not be so high that it approaches the hybridization temperature of 68°C, so the GC content should be kept <50%, and the primers should not be excessively long (>50 bases). Standard oligonucleotide software (e.g., Oligo, Primerselect) is helpful for designing primers. Primer sequences should also be checked against a database such as GenBank for any similarities to sequences in known genes.

Restriction digestion of cDNAs

It is very important that the cDNAs of both the tracer and driver sides be cut to completion before adapter ligation. If, for example, A cDNA has not digested as well as B cDNA, PCR may be biased for smaller fragments in A but not B, resulting in false positives at the end of the procedure. The A_0 and B_0 populations should be checked on a gel to ensure that the size ranges of amplified cDNAs are the same.

Monitoring subtractions

It is necessary to monitor efficiency of the subtractions to determine when to stop subtracting. In many cases a problem can be easily resolved on the spot rather than being discovered at the end of the subtractions so that it is necessary to start all over again. Subtraction efficiency can be monitored in the following ways. First, the cumulative percentage removal of tracer counts after the phenol extractions at each subtraction should be determined to provide an immediate and fairly accurate way of determining whether a particular subtraction has been successful and whether the subtraction should be repeated. Second, the degree to which A_n and B_n cross hybridize can be monitored by slot blotting cDNAs from each step of subtraction and probing the blots with the last set of subtracted cDNAs (A_n and B_n). Subtractions are generally stopped when a probe made from A_n hybridizes to the A_n cDNA pool ~20-fold better than it does to the B_n pool, and vice versa. Third, the removal or enrichment of a known differentially expressed gene in A_0 through A_n can be monitored by slot blot hybridization. If no such gene is available, then the original tracer may be spiked with some DNA such as β-galactosidase, which can be removed at the end. Fourth, if the subtractions are working, common abundant sequences should be progressively removed with each subtraction.

Anticipated Results

The end result of the procedure is the isolation of fragments of differentially expressed genes. The actual number of such genes obtained depends on the tissues being compared. Hence, if the two starting tissues are of very similar complexity, only a few genes may be obtained. On the other hand, if the tissues being compared contain a mixture of cell types and are very different, it is easily possible to obtain hundreds of differentially expressed genes. Abundant transcripts will be represented more frequently than rare transcripts. Additionally, each original transcript may be represented by multiple clones because the original cDNA was digested into fragments before subtraction. In theory, because the restriction endonucleases (*Alu*I and *Rsa*I) have 4-bp recognition sequences, digestion should produce approximately four 250-bp fragments per kilobase

original mRNA. In practice, digestion yields one to two fragments per gene. If the subtractions have been performed exhaustively, then theoretically every clone in the subtracted library should be differentially expressed. Furthermore, it should be possible to isolate genes that are 2- to 3-fold differentially expressed between two given tissues and whose abundance is as little as 5 copies mRNA/cell; however, the isolation of rare differentially expressed genes is dependent on the complexity of the starting tissues.

Time Considerations

A time schedule for this procedure is presented in Table 25B.2.3. This schedule is approximate and assumes that the procedure starts with double-stranded cDNA (see Fig. 25B.2.3).

Table 25B.2.3 Time Requirements for Preparation of Subtracted cDNA

Day	Procedure	Time required
1	*Restriction endonuclease digestion*	Overnight (0.5 hr to set up)
2	*cDNA preparation*	
	Adapter preparation	1.5 hr
	Adapter ligation	3.5 hr
	Amplification of ligated cDNA and checking by gel electrophoresis	5 hr
	Tracer and driver synthesis	Overnight (0.5 hr to set up)
3	*First (short) subtraction*	
	Tracer and driver purification and quantitation	1 hr
	Tracer and driver annealing	2 hr (short)
	Removal of annealed and ssDNA	3 hr
	Tracer and driver synthesis	Overnight (0.5 hr to set up)
4 to 6	*Second (long) subtraction*	
	Tracer and driver purification and quantitation	1 hr
	Tracer and driver annealing	40 hr (long)
	Removal of annealed and ssDNA	3 hr
	Tracer and driver synthesis	Overnight (0.5 hr to set up)
7 to 30	*Further subtractions*	Variable[a]
	Alternating short and long hybridizations	6 hr to 44 hr
	Slot blot hybridization to check the progress of subtraction	24 hr
31 to 34	*Cloning of subtracted cDNAs*	
	Amplification of subtracted cDNAs	8 hr
	Restriction endonuclease digestion	Overnight (0.5 hr to set up)
	Vector ligation	Overnight (0.5 hr to set up)
	Bacterial transformation and growth	Overnight (0.5 hr to set up)
35 to 38	*Assessment of subtracted cDNA library*	
	Growth of library	Overnight (0.5 hr to set up)
	Preparation of lifts	8 hr
	Hybridization with subtracted probes	24 hr

[a]The schedule for days 7 to 30 depends on the duration of the hybridization steps and the amount of progress with each subtraction.

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Differential Display of mRNA by PCR

BASIC PROTOCOL

This unit describes differential display to identify mRNA species for differentially expressed genes. DNA sequences corresponding to these mRNAs can be recovered, cloned, sequenced, and used for hybridization or library screening probes. This approach combines both the power of polymerase chain reaction (PCR) amplification and the high resolution of denaturing polyacrylamide gel electrophoresis for separation of amplified cDNA products. The basic principle is to reverse transcribe and systematically amplify the 3′ termini of mRNAs with a set of anchored oligo(dT) primers and an arbitrary decamer. Figure 25B.3.1 illustrates the general strategy of differential display. Specifically, an RNA sample is reverse transcribed with each of the four sets of degenerate anchored oligo(dT) primers ($T_{12}MN$), where M can be G, A, or C and N is G, A, T, and C. Each primer set is dictated by the 3′ base (N), with degeneracy in the penultimate (M) position. For example, the primer set where N = G consists of:

5′-TTTTTTTTTTTTGG-3′
5′-TTTTTTTTTTTTAG-3′
5′-TTTTTTTTTTTTCG-3′

The resulting cDNA population is PCR-amplified using the degenerate primer set, an arbitrary decamer, and radioactive nucleotide. The radioactively labeled PCR products that represent a subpopulation of mRNAs defined by the given primer set are separated on a denaturing polyacrylamide gel. By changing primer combinations, most of the RNA species in a cell may be represented. Side-by-side comparison of RNA samples from different cells allows the identification and cloning of differentially expressed genes.

Materials

Total cellular human RNA (*UNIT 4.2*) *or* poly(A)$^+$ RNA (*UNIT 4.5*)
1 U/µl human placental RNase inhibitor
10 U/µl DNase I (RNase-free)
0.1 M Tris·Cl, pH 8.3 (*APPENDIX 2*)
0.5 M KCl
15 mM $MgCl_2$
3:1 (v/v) phenol/chloroform
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
100%, 70%, and 85% ethanol
Diethylpyrocarbonate (DEPC)–treated H_2O (*UNIT 4.1*)
10 µM each degenerate anchored oligo(dT) primer set 5′-T_{12}MN-3′ (e.g., GenHunter): T_{12}MG, T_{12}MA, T_{12}MT, and T_{12}MC (M represents G, A, or C)
5× MoMuLV reverse transcriptase buffer (*UNIT 15.6*)
0.1 M dithiothreitol (DTT; *APPENDIX 2*)
250 µM and 25 µM 4dNTP mixes (*UNIT 3.4*)
200 U/µl Moloney murine leukemia virus (MoMuLV) reverse transcriptase
10× PCR amplification buffer (make as in *UNIT 15.1*, with 15 mM $MgCl_2$, but use only 0.1 mg/ml gelatin; store at −20°C)
10 µCi/µl [α-^{33}P]dATP (>2000 Ci/mmol)
2 µM arbitrary decamer (see Critical Parameters; e.g., GenHunter or Operon Technologies)
5 U/µl *Taq* DNA polymerase
Mineral oil
Formamide loading buffer (see recipe)
10 mg/ml glycogen (DNA-free)

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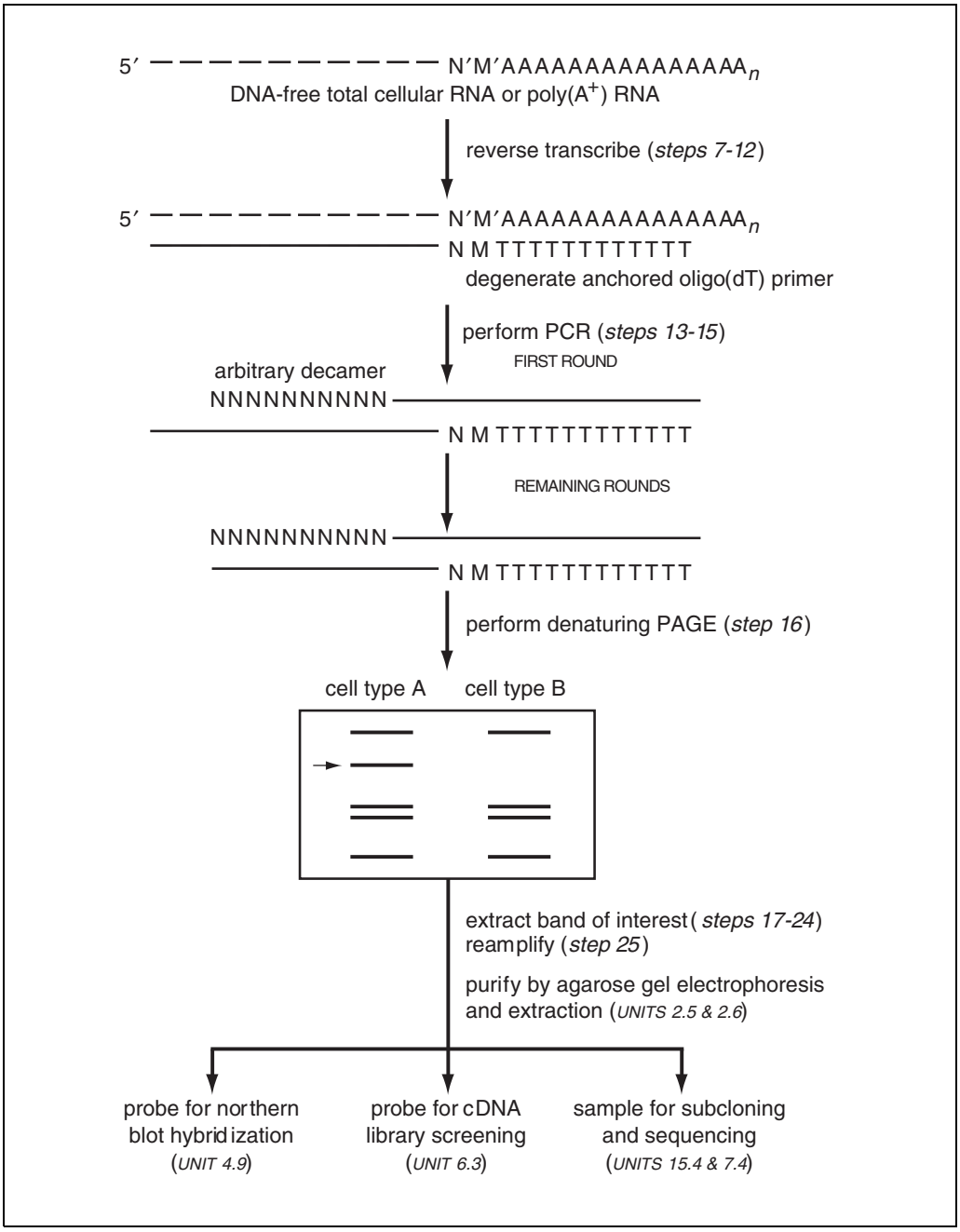

Figure 25B.3.1 Schematic representation of differential display. Diagram of gel represents results with a single primer set for two cell types, A and B. Dashed line, RNA; solid line, DNA; $T_{12}MN$, degenerate oligo(dT) primer; M indicates A, C, or G (degenerate); N can be A, C, G or T.

65°, 95°, 80°, and 100°C water baths
Thermal cycler
Whatman 3MM filter paper

Additional reagents and equipment for preparing total (*UNIT 4.2*) *or* poly(A)$^+$ (*UNIT 4.5*) RNA, quantitating RNA (*APPENDIX 3D*), PCR (*UNIT 15.1*), agarose-formaldehyde gel electrophoresis (*UNIT 4.9*), denaturing PAGE (*UNIT 7.6*), autoradiography (*APPENDIX 3A*), agarose gel electrophoresis (*UNIT 2.5A*), purifying DNA from agarose gels (*UNIT 2.6*), analysis of RNA by northern blot analysis (*UNIT 4.9*), screening libraries using oligonucleotide probes (*UNIT 6.3*), cloning PCR products (*UNIT 15.4*), and dideoxy DNA sequencing (*UNIT 7.4*)

CAUTION: This procedure should be performed only by personnel trained in the proper use of ^{33}P isotope and in NRC licensed sites. Standard precautions to prevent excessive exposure and radioactive contamination of personnel and equipment should be followed at all times.

NOTE: Experiments involving RNA require careful technique to prevent RNA degradation (*UNIT 4.1*).

Remove chromosomal DNA contamination from RNA

1. Digest DNA from total cellular RNA *or* poly(A)$^+$ RNA by mixing:

   50 µg RNA
   10 µl 1 U/µl human placental RNase inhibitor
   1 µl 10 U/µl RNase-free DNase I
   5 µl 0.1 M Tris·Cl, pH 8.3
   5 µl 0.5 M KCl
   5 µl 15 mM $MgCl_2$
   H_2O to 50 µl.

   Incubate 30 min at 37°C.

   When performing differential display, it is essential that the RNA sample be free from any genomic DNA contamination. RNA preparations isolated by various methods are often found to be contaminated with trace amounts of chromosomal DNA that results in reverse transcription–independent DNA amplification. Amounts from 15 to 100 µg of total RNA can be cleaned with this procedure.

2. Add 50 µl phenol/chloroform (3:1), vortex, and microcentrifuge 2 min at maximum speed to separate phases.

   This step serves to inactivate DNase I before cDNA synthesis during reverse transcription, so vigorous mixing is important to allow complete extraction of DNase I.

3. Transfer upper phase to a clean microcentrifuge tube and add 5 µl of 3 M sodium acetate and 200 µl of 100% ethanol. Incubate 30 min at −70°C to precipitate RNA.

4. Microcentrifuge 10 min at high speed. Remove supernatant and wash pellet (precipitated RNA) once with 500 µl of 70% ethanol.

5. Dissolve RNA pellet in 20 µl DEPC-treated water and quantitate the RNA concentration accurately by measuring the A_{260} with a spectrophotometer (*APPENDIX 3D*).

   DNA-free RNA should be stored at a concentration >1 µg/µl. It should not be diluted to the working concentration until immediately before reverse transcription. Diluted RNA should not be reused for differential display as diluted RNA is very unstable during storage and repeated freezing and thawing.

6. Check the integrity of the RNA to be used for differential display by performing agarose/formaldehyde gel electrophoresis (*UNIT 4.9*) on 3 µg of cleaned RNA. Store DNA-free RNA at −80°C until used for differential display.

   For undegraded total RNA, the 28S and 18S ribosomal RNAs should be clearly visible by ethidium bromide staining.

Reverse transcribe RNA

7. For each RNA sample, label four microcentrifuge tubes G, A, T, and C—one tube for each degenerate anchored oligo(dT) primer set.

8. Dilute 1 µg DNA-free RNA (step 5) to 0.1 µg/µl in DEPC-treated water and place on ice.

9. Set up reverse transcription of DNA-free total RNA *or* poly(A)$^+$ RNA with each of four different degenerate anchored oligo-dT primer sets (5′-T_{12}MN-3′: T_{12}MG, T_{12}MA, T_{12}MT, and T_{12}MC, where M is G, A or C) as follows:

   4 µl 5× MoMuLV reverse transcriptase buffer (1× final)
   2 µl 0.1 M DTT (10 mM final)
   1.6 µl 250 µM 4dNTP mix (20 µM final)
   0.2 µg total RNA *or* 0.1 µg poly(A)$^+$ RNA
   2 µl of one 10 µM degenerate anchored oligo(dT) primer set (T_{12}MN; 1 µM final)
   Adjust volume to 19 µl with DEPC-treated H_2O.

   There will be four reactions for each RNA sample, each made with one degenerate primer set.

10. Incubate tube 5 min at 65°C to denature the mRNA secondary structure and incubate 10 min at 37°C to allow primer annealing.

11. Add 1 µl of 200 U/µl MoMuLV reverse transcriptase to each tube, mix well, and incubate 50 min at 37°C.

12. Incubate 5 min at 95°C to inactivate the reverse transcriptase and microcentrifuge briefly at high speed to collect condensation. Place tube on ice for immediate PCR amplification *or* store at −20°C for later use (stable at least 6 months).

Perform PCR amplification

13. Prepare a 20-µl reaction mix for each primer set as follows:

   10 µl H_2O
   2 µl 10× amplification buffer (1× final)
   1.6 µl 25 µM 4dNTP mix (2 µM final)
   0.2 µl [α-^{33}P]dATP
   2 µl 2 µM arbitrary decamer (0.2 µM final)
   2 µl 10 µM degenerate anchored oligo(dT) primer set (T_{12}MN; 1 µM final)
   2 µl cDNA (step 12)
   0.2 µl 5 U/µl *Taq* DNA polymerase.

   To avoid pipetting errors, prepare enough PCR reaction mix without the arbitrary decamer for 5 to 10 reactions and aliquot 18 µl to each tube. Then add the arbitrary decamer. Otherwise it is difficult to pipet accurately 0.2 µl of Taq DNA polymerase.

14. Pipet up and down to mix well and overlay with 25 µl mineral oil.

15. Carry out PCR in a thermal cycler using the following amplification cycles:

40 cycles:	30 sec	94°C (denaturation)
	2 min	40°C (annealing)
	30 sec	72°C (extension)
1 cycle:	5 min	72°C (extension)
Final step:	indefinitely	4°C (hold).

   The 2-min incubation at 40°C is to allow sufficient time for the short primers to anneal and start extension. The short extension period at 72°C is intended to amplify only short (<600-bp) DNA products to be separated on a denaturing polyacrylamide gel.

   PCR products may be stored at 4°C until used.

16. Mix 3.5 µl PCR product with 2 µl formamide loading buffer and incubate 2 min at 80°C. Load sample onto a 6% denaturing polyacrylamide gel (*UNIT 7.6*). Run the gel ~3 hr at 60 W until xylene cyanol runs to within 10 cm of the bottom.

Flush out the urea from the gel wells with a syringe and needle just before loading samples to obtain high-resolution differential-display cDNA patterns.

Recover differentially displayed amplified DNAs

17. Carefully remove one of the glass gel plates. Place a piece of Whatman 3MM filter paper over the gel without trapping air bubbles between filter paper and gel. Dry the gel ~1 hr at room temperature without fixing it in methanol/acetic acid.

    Fixing the gel with methanol/acetic acid will make it difficult to reamplify recovered DNA because DNA is labile at acidic pH, especially at the high temperature at which the gel is normally dried.

    The dried gel should be handled with gloves to prevent DNA contamination. Always store the dried gel between two sheets of clean Whatman 3MM filter paper.

18. Use either radioactive ink or needle punches to mark X-ray film and dried gel to orient them. Expose the film 24 to 48 hr at room temperature (*APPENDIX 3A*).

19. Develop the film, align film with gel, and indicate DNA bands of interest (those differentially displayed in different lanes) either by marking beneath the film with a clean pencil or by cutting through the film.

    Typical results of differential display are shown in Figure 25B.3.2.

20. Cut out gel slice and attached Whatman 3MM filter paper with a razor blade and place in a microcentrifuge tube. Add 100 µl H_2O and incubate 10 min at room temperature.

    If more than one band is differentially expressed, extract and reamplify each one separately.

21. Cap tube tightly and boil 15 min.

    Place a lid-lock on the tube to prevent it from opening while boiling.

22. Microcentrifuge 2 min at high speed to pellet gel slice and paper debris. Decant supernatant into clean tube.

23. Add 10 µl of 3 M sodium acetate (to give 0.3 M final) and 5 µl of 10 mg/ml glycogen (as a carrier) to supernatant. Add 400 µl of 100% ethanol and incubate 30 min at −70°C. Microcentrifuge 10 min at high speed, 4°C.

    Glycogen is soluble at ethanol concentrations <85%.

24. Rinse pellet with 500 µl of 85% ethanol, air-dry, and dissolve the DNA in 10 µl H_2O.

Reamplify DNA

25. Reamplify 4 µl of the eluted DNA in a 40-µl reaction volume using the same degenerate anchored oligo(dT) primer set and PCR conditions as in steps 13 through 15, *except* add 3.2 µl of 250 µM 4dNTP mix (20 µM final) instead of 1.6 µl of 25 µM 4dNTP mix and omit isotope. Save the remaining recovered DNA at −20°C for future reamplification (stable indefinitely).

26. Electrophorese 30 µl of each PCR sample on a 1.5% agarose gel and stain with 0.5 µg/ml ethidium bromide (*UNIT 2.5A*). Store the remaining PCR samples at −20°C (stable for years).

    Most amplified DNAs should be visible after the first reamplification. Fragment molecular weights should be checked after reamplification to ensure that they are consistent with those on the denaturing polyacrylamide gel. If a DNA is not visible after the first reamplification, 4 µl of 1/100 dilution (in water) of the first reamplification sample may be used for a second 40-cycle amplification.

27. Extract the desired reamplified DNA band from the agarose gel (*UNIT 2.6*) and use it as a probe for northern blot analysis (*UNIT 6.3*) and cDNA library screening (*UNIT 6.3*).

Store extracted DNA at −20°C (stable for years) if it is not to be used immediately.

28. Characterize remaining PCR sample (from step 26) by subcloning (*UNIT 15.4*) and sequencing (*UNIT 7.4*).

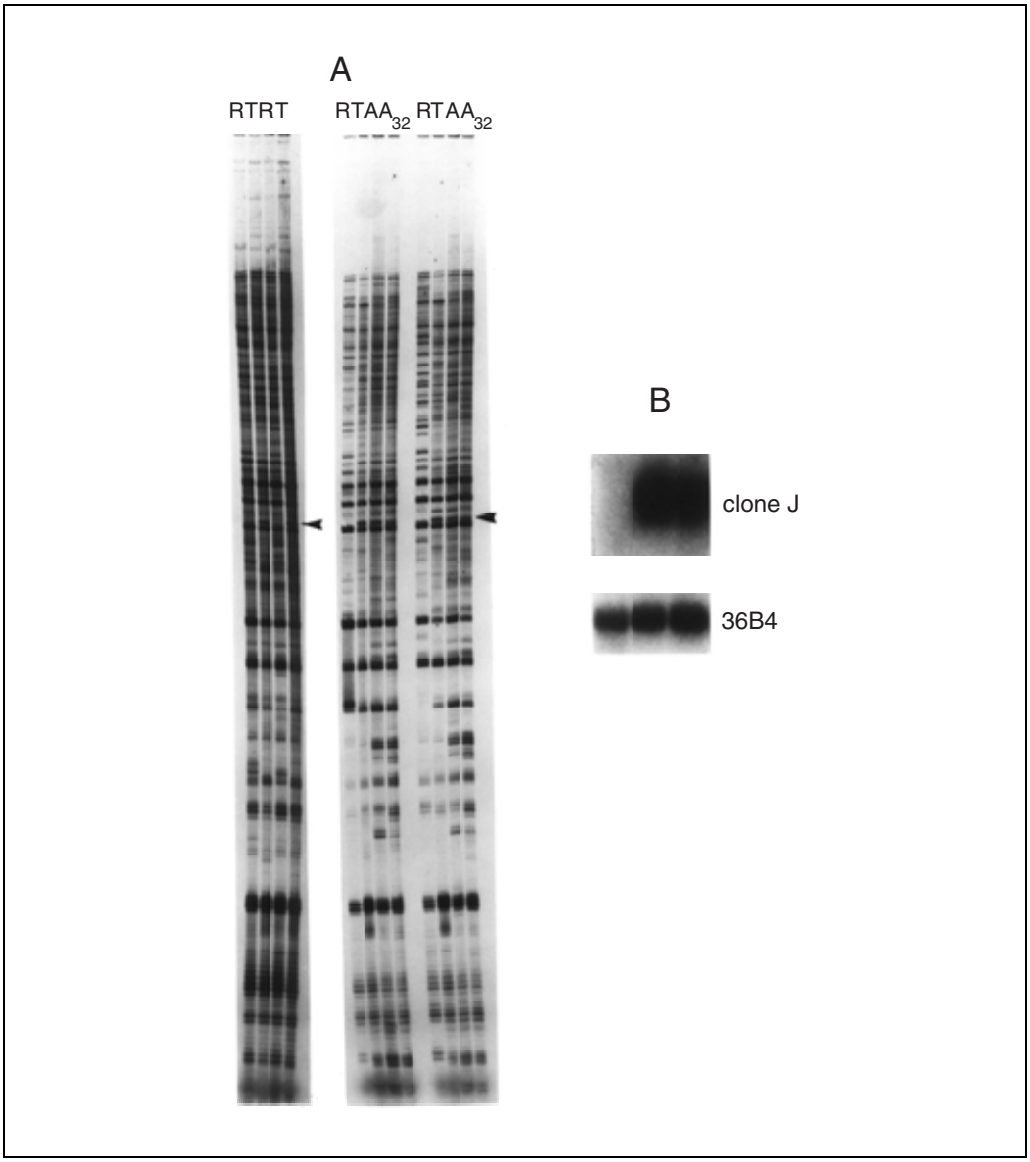

Figure 25B.3.2 Reproducibility and multiple display of mRNAs from normal versus ras/p53 mutant transformed cells. (**A**) RNA samples from normal rat embryo fibroblasts REF (R) and its ras/p53 doubly transformed derivative T101-4 cells (T) were reverse transcribed and amplified in duplicate with T12MA and OPA17 primers (left four lanes). In a separate experiment, RNA samples from REF (R), T101-4 (T), and another ras/p53 temperature-sensitive mutant transformed cell line A1-5 grown at nonpermissive temperature (A) and shifted to permissive temperature for 24 hr (A_{32}) were reverse transcribed and amplified in duplicate with T12MA and OPA17 primers (right eight lanes). An arrowhead indicates a reproducible difference between normal and transformed cells. (**B**) Northern blot analysis of this reamplified cDNA probe (named as clone J). 20 mg of total RNA from REF, T101-4, and A1-5 cells were analyzed. 36B4 was used as a probe for RNA loading control.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Formamide loading buffer

95% (v/v) formamide
0.09% (w/v) bromphenol blue
0.09% (w/v) xylene cyanol FF
Store at 4°C

COMMENTARY

Background Information

Current methods to distinguish mRNAs in comparative studies rely largely on differential or subtractive hybridization techniques (Hedrick et al., 1984; Lee et al., 1991). Several important genes implicated in tumorigenesis have been isolated using these methods (Steeg et al., 1988). Although subtraction is quite sensitive and can detect fairly rare mRNAs (see *UNIT 25B.1*), the method recovers genes incompletely and selects for genes in only one direction at a time during a two-way comparison between a pair of cells. The process is also laborious and time-consuming.

The differential display technique was developed with the goal of identifying differentially expressed genes, detecting individual mRNA species that are changed in different sets of mammalian cells, then recovering and cloning the cDNA (Liang and Pardee, 1993; Liang et al., 1993). This method utilizes polymerase chain reaction (PCR) amplification and denaturing polyacrylamide gel electrophoresis, two of the most commonly used molecular biological methods, and provides a sensitive, straightforward, and flexible approach to detect genes that are differentially expressed at the mRNA level.

In differential display, each RNA sample is first reverse transcribed with a degenerate anchored oligo(dT) primer set that anneals at the start of the poly(A) tails of mRNAs. Each degenerate anchored oligo(dT) primer set (e.g., $T_{12}MA$) will, in theory, reverse transcribe one-fourth of the total mRNA population. In combination with a decamer oligonucleotide of arbitrary sequence, which in theory can hybridize to any mRNA, cDNA fragments representing the 3′ termini of mRNAs defined by both primers are amplified. Thus, this procedure allows amplification of an mRNA subpopulation without knowledge of sequence information. If any given arbitrary decamer does not actually sample all mRNAs, different decamers can be used to permit sampling of differential mRNA populations.

Differential display can be used for many purposes. One is to provide a picture of mRNA composition of cells by displaying subsets of mRNAs as short DNA bands. This mRNA fingerprinting is useful in the same way as are two-dimensional protein gels, for example, for observing alterations in gene expression. Secondly, these DNAs can be quickly reamplified, cloned, sequenced, and compared with sequences in data banks. Finally, reamplified cDNAs can be used as probes for northern or Southern blot hybridization and to isolate genes from genomic or cDNA libraries for further molecular characterization.

Investigations of expression genetics (Sager, 1997) has gained in preeminence. The differential display procedure is being successfully employed by many research groups to identify numerous expressed genes. Related publications have increased exponentially, and currently there are ~2000. For a cross section of results see Liang et al. (1994). Thus, differential display is a viable method for the identification of novel gene targets.

Critical Parameters and Troubleshooting

The most important, powerful application of differential display is to identify and clone differentially expressed genes in various biological systems. Because the method is based on reverse transcription–PCR (RT-PCR; *UNIT 15.5*), critical parameters relevant to that procedure generally apply for this protocol.

Utilization of this technique has encountered the problem of isolation of "false-positive" transcripts—i.e., PCR products that appear to be differentially expressed but which cannot be verified when subsequent northern analysis is performed using the same RNA source. PCR is highly sensitive to minor variations in experimental procedures and is noto-

riously difficult to make quantitative. In the authors' experience, success with differential display is dependent to a large degree on experimental design, great care in achieving consistency, the use of core reagent mixes, and duplicate assays, among other things.

Many modifications of the original protocol have been described, the implementation of which have resulted in enhanced fidelity and overall utility of this evolving technique.

Isolation of RNAs that are undegraded and that are free of contaminating DNA is necessary to select optimally for expressed genes (see Quality of RNA, below). A considerable number of articles propose modifications in choice of primers for both reverse transcriptase and PCR steps. Single base oligo(dT)-anchored primers reduce the number of reactions and redundancy (Liang et al., 1994). A recent study proposes primer sequences based on frequencies of gene sequences (Pesole et al., 1998). Longer arbitrary primers seem to enhance the reproducibility of the differential display patterns (Liang et al., 1994; Zhao et al., 1995). Labelling the PCR products with [^{35}S]- or [^{33}P]deoxynucleotides has safety advantages over [^{32}P] (Trentmann, 1995). Bands may be visualized nonradioactively with silver staining or fluorescence. Improved methods for cloning differential display products have also been proposed (Comes et al., 1997; Wybranietz and Laurer, 1998).

One band on a sequencing gel often contains more than one cDNA, and the contaminating band can generate a false northern signal if its mRNA is very plentiful. For avoiding false positives, cloning strategies (Zhao et al., 1996), restriction cutting (Prasher and Weissman, 1996), nested PCR reamplification (Zhang et al., 1996; Martin et al., 1998), and single-strand conformation polymorphism gels (Miele et al., 1998) can help to avoid this problem.

Direct sequencing of differentially expressed cDNAs has been reported (Wang and Feurstein, 1995). Dot blot grids are being developed to evaluate the differential display products (Martin et al., 1998).

Recently, other methods have been developed for studying expression genetics. These include representational difference analysis (Lisitsyn, 1995), serial analysis of gene expression (SAGE; Zhang et al., 1997), and dot blot analysis (Wodicka, 1997), by which differential mRNA expression is examined with high throughput mass cDNA library screening on dot blots placed on chips, together with powerful computational analysis of sequences. This technique will in time provide massive amounts of information, although it is relatively laborious and requires special facilities.

Quality of RNA

The quality of RNA is determined by two criteria. First is the integrity of the RNA; second is the degree of chromosomal DNA contamination. The integrity of total RNA can be easily verified by agarose/formaldehyde gel electrophoresis, whereas the integrity of poly(A)$^+$ RNA must be checked by northern blot hybridization using a cDNA probe for an mRNA with known molecular weight. Contamination by chromosomal DNA can be checked by performing differential display omitting the reverse transcription step. Under the PCR conditions used for differential display (i.e., low dNTP concentrations), RNA amplification is dependent on reverse transcription. Because total RNA isolated with various methods is generally found to be contaminated with DNA, it is recommended that, as a good practice, RNA samples be treated with DNase I before being used for differential display.

Design of arbitrary decamers

Generally any arbitrary decamer can be used as long it does not contain palindromic sequences and has a G+C content of 50% to 70%. The original decamer chosen for this application was from the mouse thymidine kinase gene (Liang and Pardee, 1993), but it has been used successfully to detect multiple mRNAs in cells of various species. Because the arbitrary decamers have been shown to contain up to 4-bp mismatches with the original cDNA templates and these mismatches are often clustered at the 5′ end of the primers (Liang et al., 1993), the arbitrary decamers can be designed in such a way that the 3′ sequences are maximally randomized while the 5′ bases (up to four bases) are fixed. The G+C content of the arbitrary decamers can be increased or decreased to reflect the G+C content of the genome of the organism from which the mRNA is isolated.

False-positive difference

The intrinsic problem encountered with differential display, as with any method based on PCR, is that it is highly sensitive to minor variations. True differences in expression must be differentiated from the "noise" that is the major source of false-positive differences. If a pair of RNA samples is to be compared, the displayed (DNA) pattern differences must be reproducible. An advantage of differential dis-

play is the ability to simultaneously compare more than two relevant RNA samples (e.g., from different cell types or stages of development); multiple display thus has a built-in internal control for distinguishing "noise" from true differences. This also facilitates isolation of genes that really give useful results for the system under study.

Size of DNA probe

Short DNA probes (<150 bp) have been found to be hard to label and often fail to produce any signals in northern blot hybridizations. Therefore, it is advised that only DNA bands >150 bp be further characterized by northern blot analysis and that smaller bands be ignored.

Anticipated Results

This method should produce reproducible amplified DNA patterns. The reproducible DNA bands representing differentially expressed genes should be readily reamplifiable and usable as probes for northern blot analysis or cDNA library screening.

Time Considerations

The whole procedure from RNA to DNA samples ready to use as probes can be performed within three days. Treating RNA with DNase I and checking its integrity by gel electrophoresis takes ~2 hr. Reverse transcription takes ≤2 hr. Setting up 40 PCR samples requires 1 to 2 hr. PCR amplification requires ~4 hr but can be performed overnight. Preparing, running, and drying the denaturing polyacrylamide gel takes 1 day. Autoradiography can be as brief as overnight. Recovery, reamplification of DNA, and extraction of reamplified DNA from an agarose gel can easily fit into the third day. Northern blot analysis requires an additional 2 days.

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Key Reference

Liang et al., 1993. See above.

Uses the protocol outlined here and presents examples of data generated.

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Restriction-Mediated Differential Display (RMDD)

Restriction-mediated differential display (RMDD) can be applied to identify differentially expressed (i.e., up- or down-regulated) genes in many eukaryotic cells or tissues by comparison of band patterns obtained from two or more different RNA preparations. As opposed to early differential display or other RNA-fingerprinting protocols based on arbitrarily primed PCR, RMDD provides very robust and reproducible results which are largely independent of the exact amount of input material or of the exact cycling conditions, respectively. Two different PCR strategies for fragment amplification, depending on the complexity of the material under investigation as well as the appropriate choice of the restriction enzyme or enzymes used, are discussed (see Strategic Planning). The first protocol describes oligo(dT)-primed conversion of total RNA into double-stranded cDNA, which is cleaved with a frequently cutting restriction enzyme, ligated to linker molecules (thus creating the "RMDD library"), and amplified with labeled selective 3′-elongated oligonucleotide primers to generate subpools of amplified fragments which represent the 3′-ends of the cDNA molecules (see Basic Protocol and Fig. 25.B4.1). A protocol outlining two-phase PCR is given as an alternative to the amplification steps used in the Basic Protocol (see Alternate Protocol). This protocol is usually chosen if the RNA samples to be analyzed are particularly complex. The final protocol describes nonradioactive fragment analysis through the use of biotinylated primers and direct-blotting electrophoresis (see Support Protocol).

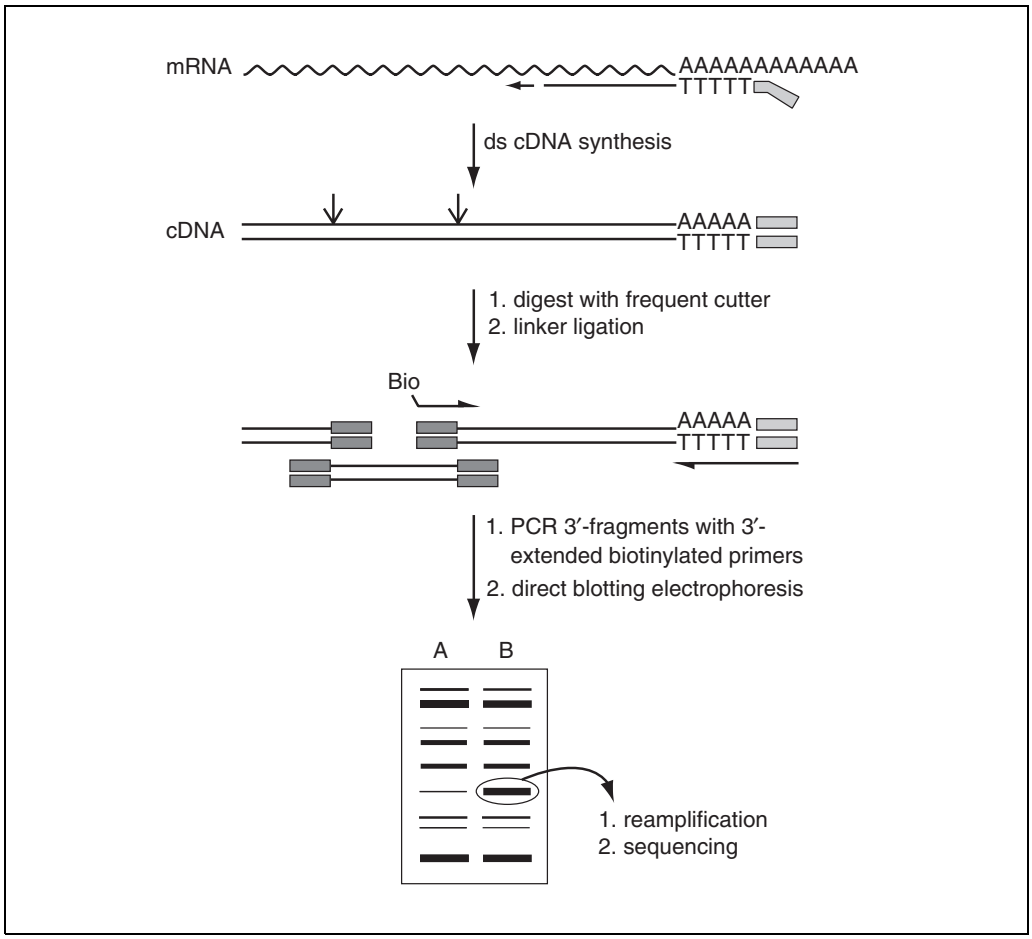

Figure 25B.4.1 Schematic of RMDD.

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NOTE: 5′-labeled primers are indicated by an asterisk (*). The label can be a radioactive isotope (e.g., ^{33}P) or a nonradioactive label such as biotin or digoxigenin. In the latter case, labels should be attached via a sufficiently long spacer to the oligonucleotide (e.g., tetraethylene glycol from Eurogentec) to ensure maximum detection sensitivity.

NOTE: The technology described in this unit is protected by certain patent rights (US 5,876,932; EP 0 743 367; JP 96/308598). Commercial application of RMDD (including in-house research projects of any company) thus requires a license. No license is required for academic use.

STRATEGIC PLANNING

The "RMDD library" contains a mixture of restriction fragments of all cDNA molecules obtained from the respective biological sample. It has been estimated that a single cell type contains ~10,000 different mRNA molecules, resulting in 10,000 different cDNA species. For successful gel display of the fragments derived from the 3′ ends of these cDNA molecules, a strategy must be provided to subdivide this rather complex fragment mixture into a number of subpools, each containing a sufficiently low number (i.e., ≤50 to 100) of different fragment species. This can be easily achieved by fragment amplification employing oligonucleotide primers each carrying one additional "selective" base at the 3′-end. Theoretically, such a selective base allows primer extension by a polymerase only if it perfectly matches the corresponding base on the other strand. Combining selective primers directed against the ligated linker and against the sequence introduced by the cDNA primer thus allows subdivision of fragments into nonoverlapping subpools. The RMDD protocol (see Basic Protocol and Alternate Protocol) involves two subsequent rounds of amplification, the first employing selective primers extended by one base each and the second employing primers extended by one more base, providing a total number of $12 \times 16 = 192$ reactions to be performed for complete coverage of all generated 3′-end fragments. Two rounds are chosen, since the discrimination of a polymerase against extension of primers distinguished by a mismatch at the second last position is much less pronounced than the discrimination against extension of terminal mismatches, prohibiting use of primers carrying two selective bases at their 3′-end in a single round of PCR. However, in practice, a certain extent of "bleedthrough" can still be observed (i.e., amplification of fragments with a given selective primer, which theoretically should not take place due to a 3′-terminal mismatch of the annealed primer).

If mRNA complexity is not too high (e.g., material obtained from cell cultures or "simple" tissues of low complexity), this "bleedthrough" usually does not cause any problems; nevertheless, when working with highly complex samples (e.g., RNA isolated from mammalian brain), bleedthrough may render band patterns too crowded for reliable isolation of particular bands of interest. To reduce bleedthrough, both first and second amplification reactions can be performed in a "two-phase" manner (see the Alternate Protocol). The first phase, performed at extremely low concentrations of dNTPs (i.e., 2 μM each), involves 10 (first amplification) or 15 (second amplification) cycles and defines which products will be amplified to a detectable level. This phase exploits the fact that mismatch extension can be significantly reduced at low dNTP levels. For the second phase, dNTP concentrations are raised to "normal" levels (i.e., 200 μM each), which, after an additional 10 cycles, allows for accumulation of the desired amount of product.

The choice of the restriction enzyme used for RMDD depends on the particular organism to be analyzed, since average fragment size may vary due to differences in codon usage and G/C content. To obtain cDNA 3′-fragments in a size range optimal for gel display (i.e., most of the fragments having a size between 100 and 700 bp), an appropriate enzyme

has to be employed—e.g., *Mbo*I, as described in this protocol (see Basic Protocol)—which has proven satisfactory with RNA isolated from man, rat, mouse, corn, and Arabidopsis. Should another enzyme be chosen, linker and linker primer sequences will have to be modified accordingly, and the same holds true if, for the sake of more complete coverage of the transcriptome, experiments are repeated with a second enzyme. Computer analysis has demonstrated that in man and rodents roughly 80% to 85% of all transcripts contain a recognition site for *Mbo*I (unpub. observ.); therefore, 15% to 20% of transcripts would be inaccessible to analysis using this particular enzyme. Accordingly, if nearly complete coverage of transcripts is desired, a second-pass RMDD analysis might be performed with a second frequently cutting enzyme. Performing RMDD with a second enzyme, assuming both enzymes recognize 80% of cDNAs each, would provide a total coverage of 96% of all transcripts.

RMDD LIBRARY PREPARATION AND TWO-ROUND AMPLIFICATION

BASIC PROTOCOL

This protocol describes conversion of total RNA to labeled PCR products, which are ready to be displayed by gel electrophoresis.

Materials

50 µg total RNA (*UNITS 4.1 & 4.2*)
RNase-free water
10 µM cDNA primer CP29V: 5′-ACC TAC GTG CAG ATT TTT TTT TTT TTT TX_1-3′ (X_1 = A, C, or G; equimolar amounts of all three species; see *UNIT 2.11* for oligonucleotide synthesis)
100 mM RNase-free DTT (Life Technologies)
5× SuperScript buffer (Life Technologies)
10 mM RNase-free and standard dNTPs
40 U/µl RNase inhibitor (e.g., RNasin)
200 U/µl SuperScript II reverse transcriptase (Life Technologies)
5× second-strand buffer II (*UNIT 5.5*)
1.5 U/µl RNase H
10 U/µl *E. coli* DNA polymerase I
Phenol equilibrated with TE buffer, pH 8.0 (*UNIT 2.1A*)
Chloroform
20 mg/ml glycogen
28% PEG 8000/3.6 mM $MgCl_2$ (see recipe)
70% and 100% ethanol
10× universal buffer (Stratagene)
4 U/µl *Mbo*I restriction endonuclease (Stratagene)
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
10 mM ATP
0.5 µg/µl *Mbo*I-linker ML2025 (see recipe)
T4 DNA ligase and 10× buffer (Roche)
1× and 0.25× TE buffer, pH 8.0 (*APPENDIX 2*)
4 µM primer CP28X_1: 5′-ACC TAC GTG CAG ATT TTT TTT TTT TTT TX_1-3′ (X_1 = A, C, or G; see *UNIT 2.11* for oligonucleotide synthesis)
4 µM primer ML19Y_1: 5′-TGC TAA GTC TCG CGA GAT CY_1-3′ (Y_1 = A, C, G, or T; see *UNIT 2.11* for oligonucleotide synthesis)
10× PCR buffer (see recipe)
20 mM $MgCl_2$ (*APPENDIX 2*)
RediLoad (Research Genetics)
5 U/µl *Taq* DNA polymerase
100-bp DNA size ladder (e.g., Life Technologies)

1.5% agarose gel (*UNIT 2.5A*)
4 µM primer CP28X_1X_2: 5′-ACC TAC GTG CAG ATT TTT TTT TTT TTT T X_1X_2-3′ (X_2 = A, C, G, or T; see *UNIT 2.11* for oligonucleotide synthesis)
4 µM labeled primer *ML18Y_1Y_2: 5′-*GCT AAG TCT CGC GAG ATC Y_1Y_2-3′ (Y2 = A, C, G, or T; see *UNIT 2.11* for oligonucleotide synthesis)
Formamide buffer: 5 mM EDTA/0.1% bromophenol blue in 99% deionized formamide

22°, 37°, 42°, 65° and 75°C water bath, heat blocks, or equivalent
Thermal cycler with heated lid
96-well PCR plates (e.g., MJ Research)

Additional reagents and equipment for ethanol precipitation and phenol/chloroform extraction of DNA (*UNIT 2.1A*), and pouring and running (*UNIT 2.5A*) agarose and 6% polyacrylamide gels (*UNIT 7.6*)

Synthesize first-strand cDNA

1. Ethanol precipitate 50 µg total RNA (*UNIT 2.1A*) and dissolve in 15.5 µl RNase-free water. Add 1.5 µl of 10 µM cDNA primer CP29V, denature 5 min at 65°C (e.g., in a heat block), and cool down on ice.

   It is not necessary to isolate poly(A+) RNA. Band patterns obtained with mRNA are virtually identical to those obtained with total RNA. On the other hand, mRNA isolation is a potential source of variation and should therefore be avoided.

   DEPC treatment will not usually be required for RNase-free water.

2. Assemble components for first-strand synthesis on ice (29.1 µl total):

   17.0 µl freshly denatured RNA with cDNA primer
   3.0 µl 100 mM RNase-free DTT
   6.0 µl 5× SuperScript buffer
   1.5 µl 10 mM RNase-free dNTPs
   0.6 µl 40 U/µl RNase inhibitor (e.g., RNasin)
   1.0 µl 200 U/µl SuperScript II reverse transcriptase.

   Mix well and incubate 1 hr at 42°C. Stop reaction by placing on ice.

   Incubation is best done in a water bath or thermal cycler. Hot air ovens do not guarantee sufficiently quick heating of samples.

   To check for possible RNA degradation in the course of first-strand synthesis due to RNase contamination, 0.5 to 1 µl of the first-strand synthesis reaction can be analyzed on a 1% standard agarose gel (UNIT 2.5A; no special RNA gel is required), watching for undegraded ribosomal RNA bands.

Synthesize second-strand cDNA

3. Assemble on ice the following components (207.2 µl total) for second-strand synthesis:

   48 µl 5× second-strand buffer II
   3.6 µl 10 mM dNTPs
   148.4 µl H_2O
   1.2 µl 1.5 U/µl RNase H
   6.0 µl 10 U/µl *E. coli* DNA polymerase I.

4. Combine first-strand and second-strand synthesis reactions. Mix and incubate for 2 hr at 22°C. After completion of second-strand synthesis, inactivate DNA polymerase by heating for 20 min to 75°C.

Purify cDNA

5. Extract with 100 µl phenol equilibrated with TE buffer, pH 8.0. Extract again with 100 µl chloroform.

   UNIT 2.1A describes the procedures for phenol-chloroform extraction of DNA.

   CAUTION: *Phenol and chloroform are severe health hazards. See UNIT 2.1A for precautions.*

6. For size-selective PEG precipitation, carefully mix:

   200 µl phenol/chloroform–extracted ds cDNA
   1.0 µl 20 mg/ml glycogen
   200 µl 28% PEG 8000/3.6 mM $MgCl_2$.

   Let the reaction (401 µl total) stand at room temperature for 5 min, then microcentrifuge for 15 min at maximum speed, 10°C. Wash pellet carefully with 70% ethanol.

   This precipitation step removes unincorporated cDNA primer as well as small (i.e., below ~100 nt) nucleic acid molecules. Since size-selective PEG precipitations are susceptible to minor concentration changes, it is imperative to adhere to the following guidelines:

   1. Make sure to pipet exactly 200 µl ds cDNA. Vapor pressure of chloroform dissolved in the aqueous phase tends to displace liquid from the pipet tip, making accurate pipetting difficult. One way to overcome this problem is to repeatedly (5 to 10 times) withdraw and expel again ~50 to 100 µl of the chloroform-saturated aqueous phase before pipetting the required 200 µl, thus allowing the pipet to saturate with chloroform vapor.

   2. The 28% PEG/3.6 mM $MgCl_2$ solution is rather viscous. Pipet slowly and carefully, again being sure to accurately transfer the required volume.

   3. Mix carefully by first repeatedly inverting the tube, then vigorously vortexing. Due to viscosity, complete and homogeneous mixing takes a while.

   During addition of PEG solution, a white glycogen precipitate usually forms. This becomes invisible again in the course of mixing.

   When washing the pellet with ethanol, detachment from the tube wall does no harm since the pellet is too large to be easily lost.

Perform restriction digest

7. Dissolve the pellet on ice in the following solution (96 µl total):

   15.0 µl 10× universal buffer
   81.0 µl H_2O.

   Instead of the Universal buffer supplied by Stratagene, any buffer supplied with the restriction enzyme can be used. In this case, adhere to the manufacturer's recommendations concerning dilution of buffer stock.

8. Add 4.0 µl of 4 U/µl *Mbo*I and incubate 1 hr at 37°C. Inactivate the enzyme by heating 20 min at 65°C.

   The choice of restriction enzyme is discussed elsewhere in this unit (see Strategic Planning).

9. Extract with 50 µl phenol buffered with TE buffer, pH 8.0, then with 50 µl chloroform. Add 1 µl glycogen and 10 µl 3 M sodium acetate, pH 5.2, followed by 2.5 vol 100% ethanol. Microcentrifuge 20 min at maximum speed and wash pellet with 70% ethanol. Air dry pellet briefly (5 to 10 min). Do not apply heat and/or vacuum, since overdrying DNA pellets might make resuspending them difficult.

Perform linker ligation

10. Dissolve pellet in ligation mix (20 µl total), consisting of the following components:

    1.2 µl 10× ligation buffer
    2.0 µl 10 mM ATP
    8.0 µl 0.5 µg/µl *Mbo*I-linker ML2025
    7.8 µl H_2O
    1.0 µl 1 U/µl T4 DNA ligase.

    Ligate overnight at 16°C or over the weekend at 4°C.

11. Add 90 µl water, mix, and extract with 50 µl phenol buffered with TE buffer, pH 8.0, then with 50 µl chloroform. For removal of unligated linkers, assemble a second PEG precipitation reaction (201 µl total):

    100 µl phenol-extracted ligation products
    1.0 µl glycogen
    100 µl 28% PEG/3.6 mM $MgCl_2$.

    Let stand at room temperature 5 min, then microcentrifuge 15 min at maximum speed, 10°C. Wash pellet carefully with 70% ethanol and resuspend in 40 µl TE buffer, pH 8.0.

    For precautions, see step 6.

Perform first-round amplification of 3′-cDNA fragments

12. Set up first-round amplification reactions by combining 1 µl of each of the three 4 µM $CP28X_1$ (X_1 = A, C, or G) primers with 1 µl of each of the four 4 µM $ML19Y_1$ (Y_1 = A, C, G, or T) primers in separate tubes on ice (12 reactions total). Assemble a master mix with all remaining components (recipe is for 1 reaction):

    2.0 µl template (PEG-precipitated ligation products)
    2.0 µl 10× PCR buffer
    1.5 µl 20 mM $MgCl_2$
    0.4 µl 10 mM dNTPs
    2.0 µl RediLoad
    9.9 µl H_2O
    0.2 µl 5 U/µl Taq DNA polymerase.

    Assemble reactions and place the tubes in the wells of a thermocycler preheated to 90°C.

13. Apply the following cycling program:

Initial step:	1 min	94°C (denaturation)
25 cycles:	20 sec	94°C (denaturation)
	30 sec	65°C (primer annealing)
	4 min	72°C (primer extension)
Final step:	indefinitely	10°C (hold/extension).

14. Load 10 µl of each reaction onto a 1.5% agarose gel and check for successful amplification by agarose gel electrophoresis (*UNIT 2.5A*). Include a 100-bp ladder as a size marker.

    PCR conditions are adjusted in such a way that the amount of primers limits the amount of product. The long extension time ensures that differently sized products are simultaneously amplified essentially without a bias against the longer ones. Agarose gel electrophoresis should yield smears between ~100 bp and ~700 bp with very few (if any) discrete bands being visible. Most importantly, reactions obtained with the same primer combina-

tion, but from different RNA samples to be compared, should look essentially indistinguishable. If appearance and/or amount of material should visibly differ, probably one of the enzymatic steps prior to amplification was performed at too low an efficiency (see Troubleshooting and Table 25.B4.1).

15. Dilute reactions 1:100 with 0.25× TE buffer, pH 8.0.

    Diluted reactions can be indefinitely stored at −20°C.

Perform second-round amplification of 3′-cDNA fragments

16. Set up second-round amplification mix by combining in 96-well plates each of the 12 $CP28X_1X_2$ primers with each of the 16 $^{*}ML18Y_1Y_2$ primers (192 different reactions per sample; 20 µl each):

    2.0 µl template (diluted first-round PCR)
    2.0 µl 10 × PCR buffer
    1.5 µl 20 mM $MgCl_2$
    0.4 µl 10 mM standard dNTPs
    2.0 µl 4 µM primer $CP28X_1X_2$ (X_2 = A, C, G, or T)
    2.0 µl 4 µM labeled primer $^{*}ML18Y_1Y_2$ (Y_2 = A, C, G, or T)
    2.0 µl RediLoad
    7.9 µl H_2O
    0.2 µl 5 U/µl *Taq* DNA polymerase.

    Make sure that for every reaction, X_1 and Y_1 of the second-round amplification are identical to X_1 and Y_1 of the first-round amplification. PCR can be conveniently performed in two 96-well plates per RNA sample.

    It is highly preferable to use a thermocycler equipped with a hot top, obviating the need to cover reactions with oil.

    Use of labeled primers instead of incorporating labeled nucleotides has the advantage that (1) only one of two complementary strands is visualized, thus limiting complexity of band patterns (usually, two complementary strands of equal length show slightly different mobility in polyacrylamide gels), and (2) label intensity does not increase with fragment length. In addition, if biotin is used as a label, incorporation of an undefined number of biotin molecules (it is not possible to replace all nucleotides of a given type by enzymatic incorporation of the biotinylated analog) into the amplified strands leads to pronounced smearing of the obtained bands due to the incremental mobility shift caused by each of the biotin groups in a DNA molecule.

17. Apply the cycle program of step 13, but for only 20 cycles. Check for successful amplification by agarose gel electrophoresis (also see step 13).

    Reactions obtained with the same primer combination but from different RNA samples should again look essentially indistinguishable, whereas reactions obtained with different primer combinations usually look distinct. Other than with first-round PCR products, usually a small number of discrete bands (e.g., 1 to 5) can be observed.

18. Transfer 5 µl of each reaction into a fresh microtiter plate containing 5 µl formamide buffer per well and mix. Denature 2 min at 75°C.

    Nonradioactively labeled PCR products in formamide buffer can be stored for several months at −20°C. When radioactive labeling is chosen, storage time is limited by decay of the incorporated isotope.

Label nucleotides

19a. *For radioactive labeling:* Load 1 to 2 µl sample into the slots of a denaturing 6% polyacrylamide gel and run as described in *UNIT 25B.3*, starting at Basic Protocol, step 16, of that unit.

19b. *For nonradioactive labeling and use of direct blotting electrophoresis:* See Support Protocol, below.

ALTERNATE PROTOCOL

AMPLIFICATION BY TWO-PHASE PCR

Alternatively, the amplification steps (see Basic Protocol, steps 12 to 17) can be replaced by a two-phase PCR (see Strategic Planning). This procedure decreases the "bleedthrough" sometimes observed between different PCRs obtained from the same sample by "illegitimate priming" (i.e., priming with a mismatch at the primer's 3′-ultimate base). The approach is to perform the first 10 to 15 cycles of each PCR at an extremely low nucleotide concentration (2 μM each), which increases the bias of *Taq* polymerase against mismatch extension. After these initial cycles are finished and the product composition in each reaction has been defined, reactions are supplemented with nucleotides to a final concentration of 200 μM each, thus allowing sufficient amounts of amplification products to be generated. The drawback is the increase in hands-on time required for pipetting.

Additional Materials *(also see Basic Protocol)*

0.1 mM dNTPs (freshly diluted from 10 mM dNTPs)

Perform first-round low-concentration amplification

1. Synthesize ds cDNA (see Basic Protocol, steps 1 to 11).
2. Set up first-round 2-μM amplification reactions (12 different reactions per sample, 20 μl each):

   2.0 μl template (PEG-precipitated ligation products; see Basic Protocol, step 11)
   2.0 μl 10× PCR buffer
   1.5 μl 20 mM $MgCl_2$
   0.4 μl 0.1 mM dNTPs (freshly diluted from 10 mM dNTPs)
   2.0 μl 4 μM primer CP28X_1 (X_1 = A, C, or G)
   2.0 μl 4 μM primer ML19Y_1 (Y_1 = A, C, G, or T)
   9.9 μl H_2O
   5 U/μl 0.2 μl *Taq* DNA polymerase.

   Again, all PCR mixtures should be prepared as master mixes.

3. Carry through the same program as described (see Basic Protocol, step 13), except for 15 rather than 25 cycles.

Perform first-round normal-concentration amplification

4. Transfer reaction tubes to ice. To each tube add 20 μl of 200 μM amplification mix, prepared as follows:

   2.0 μl 10 × PCR buffer
   1.5 μl 20 mM $MgCl_2$
   0.8 μl 10 mM dNTPs
   4.0 μl RediLoad
   11.5 μl H_2O
   0.2 μl 5 U/μl *Taq* DNA polymerase.

5. Repeat the program cycle as described (see Basic Protocol, step 13), performing the remaining cycles (i.e., 16 to 25).

6. Check products by agarose gel electrophoresis (see Basic Protocol, step 14 and *UNIT 2.5A*).

7. Dilute reactions 1:100 with 0.25× TE buffer.

Perform second-round low-concentration amplification

8. Using 96-well microtiter plates, set up second-round 2 µM amplification reactions (192 different reactions per sample; 20 µl each):

   2.0 µl template (diluted first-round PCR)
   2.0 µl 10× PCR buffer
   1.5 µl 20 mM $MgCl_2$
   0.4 µl 0.1 mM dNTPs
   4.0 µl 4 µM primer $CP28X_1X_2$ (X_2 = A, C, G, or T)
   4.0 µl 4 µM labeled primer $^*ML18Y_1Y_2$ (Y_2 = A, C, G, or T)
   5.9 µl H_2O
   5 U/µl 0.2 µl *Taq* DNA polymerase.

9. Transfer plates to the preheated wells of a thermal cycler and cycle as described above (see Basic Protocol, step 13), except for only 10 rather than 25 cycles.

Perform second-round normal-concentration amplification

10. Cool reaction tubes on ice and add 20 µl of 200 µM amplification mix (step 4) to each tube.

11. Repeat the program (see Basic Protocol, step 13), this time using 20 cycles (i.e., add 10 more cycles).

12. Check products by agarose gel electrophoresis (see Basic Protocol, step 14 and *UNIT 2.5A*).

Agarose gel electrophoresis can be skipped if radioactive label is used. In the latter case, adhere to the usual precautions for working with radioisotopes (APPENDIX 1F) and handle samples at a dedicated workspace only.

DIRECT BLOTTING ELECTROPHORESIS

SUPPORT PROTOCOL

The authors have found direct blotting electrophoresis (DBE) to be an extremely helpful technique to get high-quality display results from amplified RMDD products and to simplify physical access to bands of interest. In contrast with standard fragment analysis (see Chapter 2) based on radioactive labeling, it is not necessary, for the sake of optimal resolution of different size ranges, to perform "short" and "long" runs of each sample. In DBE, all fragments, including the largest ones, pass the whole length of the gel before being transferred to the blotting membrane, providing unsurpassed resolution of bands in the size range relevant for RMDD. Working with nonradioactive materials provides considerable convenience, and stained bands can be directly cut out of the blotting membrane for recovery and analysis.

Additional Materials *(also see Basic Protocol)*

TBE electrophoresis buffer (*APPENDIX 2*) standard and degassed (i.e., stirred under vacuum 20 min)
Maleic buffer, pH 7.5 (see recipe)
1.5% blocking reagent (see recipe)
Streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals)
Reaction buffer, pH 9.5 (see recipe)
NBT/BCIP in 67% (v/v) DMSO (Roche Molecular Biochemicals)

Primers (see *UNIT 2.11* for oligonucleotide synthesis):
CP28: 5′-ACC TAC GTG CAG ATT TTT TTT TTT TTT T-3′
ML18: 5′-GCT AAG TCT CGC GAG ATC-3′

GATC 1500 Direct Blotting Electrophoresis System (GATC Biotech AG)
Direct blotting membrane (GATC Biotech AG)
10-ml syringe and 25-G needle
32-well sharkstooth comb
GELoader tips (Eppendorf) with capillary-like part cut away
Stratalinker (Stratagene)
Developing drum (e.g., GATC tube; GATC Biotech AG)
Adhesive tape
Rolling incubator accepting 18 × 35–cm tubes and capable of revolving at ~20 rpm
2-mm-thick polyethylene wrap (e.g., Neolab, Heidelburg, FRG) or material from a thick hybridization bag
T-A cloning system (e.g., Invitrogen; optional)

Additional reagents and materials for casting denaturing polyacrylamide gels (*UNIT 2.12*), agarose gel electrophoresis (*UNIT 2.5A*), and molecular cloning of PCR products (*UNIT 15.7*).

NOTE: For details concerning use of the GATC 1500 Direct Blotting Electrophoresis apparatus, consult the manufacturer's instructions.

Prepare the gel

1. Cast a denaturing 4.5% polyacrylamide gel (*UNIT 2.12*). Attach a 40- to 45-cm long piece of blotting membrane to the conveyor belt of the direct blotting electrophoresis system. Mount the gel on the apparatus and fill with the appropriate amount of TBE electrophoresis buffer, using degassed buffer in the lower chamber. Move the leading edge of the membrane 1 cm past the lower edge of the gel. Connect apparatus to a high-voltage power supply.

   When choosing the direct blotting technique, all fragments, including the largest ones, pass through the whole length of the gel. Thus, a lower acrylamide concentration (i.e., 4.5% instead of 6%) is used as compared to the concentration used for standard sequencing gels.

2. Prerun (i.e., with no sample) the gel for 30 min with the power supply set to 2000 V and 30 W as limiting parameters.

Electrophorese samples and transfer to the membrane

3. Rinse gel slot with TBE buffer using a 10-ml syringe and 25-G needle, and insert a 32-well sharkstooth comb. Using GELoader tips with the capillary-like part cut away, load 1 to 1.5 µl denatured reaction (see Basic Protocol, step 18) per well, being sure to load the whole gel within ~10 min.

   Although 48-well combs are available as well, no satisfactory results could be obtained with them in the authors' laboratory.

   Do not use the first and the last slot of a gel, since the corresponding lanes easily run off the membrane due to imprecise membrane alignment prior to the run.

4. Start electrophoresis with the same parameters used for prerunning. After 45 to 50 min, start the conveyor belt with an initial speed of 16 cm/hr, linearly decreasing to 10 cm/hr.

   The continuous decrease in conveyor belt speed (i.e., in the blotting membrane feed rate) compensates for the nonlinear mobility of differently sized DNA molecules. The chosen parameters yield an approximate equidistant spacing of bands of different size (e.g., the

distance between a 100- and a 150-bp band is roughly the same as the distance between a 400- and a 450-bp band).

At the end of the run, the conveyor belt and membrane are wound up to the back roller. The membrane can be left wound up for drying overnight. Alternatively, it can be removed and hung up in a dust-free space.

If a size marker is desired, biotinylated Sequamark 10-bp ladder (Research Genetics) turns out to be optimal. This marker provides an accurate and easily identifiable standard for DNA fragments up to 500 bp; however, 5- to 10-fold concentration of the marker by precipitation is necessary to obtain sufficient sensitivity.

5. Air-dry the membrane overnight and fix by gentle UV irradiation in a Stratalinker with a UV dosage of ~10,000 μJ/cm^2 (i.e., ~$^1/_{10}$ the "auto-cross-link" dosage).

   For later recovery of bands of interest, it is important not to overfix membranes.

Rinse membrane and block nonspecific binding

6. Insert membrane into a suitable developing drum (e.g., GATC tube), fix with some adhesive tape, and rinse with 100 ml water while rotating 5 min on a suitable rolling incubator.

   Any roller that accepts a tube 18 cm in diameter × 35 cm long and is able to revolve at ~20 rpm will do.

7. Replace water with 150 ml maleic buffer, pH 7.5, and equilibrate membrane by rotating another 5 min. Pour buffer into a beaker and store for later use.

8. Incubate 40 to 50 min in a rolling incubator with 80 ml of 1.5% blocking reagent.

Label bands with streptavidin-alkaline phosphatase

9. Discard buffer and add 20 ml of 1.5% blocking reagent and 2 to 4 μl streptavidin–alkaline phosphatase conjugate. Incubate membrane 30 min in a rolling incubator.

10. Pour off buffer completely and wash 5 min, using the 150 ml maleic buffer set aside in step 7. Replace with 150 ml fresh maleic buffer and wash 10 min. Replace with another 150 ml maleic buffer and wash 15 min.

11. Replace with 150 ml reaction buffer, pH 9.5, and equilibrate membrane 5 min.

Develop color

12. For color development, pour off buffer and add 20 ml reaction buffer containing 400 μl NBT/BCIP stock solution. Develop under slow rotation for 2 to 3 hr.

    CAUTION: *NBT is a suspected carcinogen. Moreover, the DMSO in the concentrated stock solution might mediate penetrance of dissolved substances through the skin, and is itself hazardous. Wear gloves, replace contaminated gloves immediately, and carefully avoid any skin contact. Dispose of according to institutional regulations (also see* APPENDIX 1H*).*

13. Pour off developing solution and perform three 10-min rinses with 150 ml water each.

14. Put the wet membrane between two sheets of 2-mm-thick polyethylene wrap or material from a thick hybridization bag. Inspect wet membranes visually for bands appearing significantly stronger or weaker in one lane as compared to adjacent corresponding lanes.

    Polyethylene wrap is also called "tubular film" and must be thick, as thinner material makes handling of the wrapped membranes much more difficult and might not be a sufficient barrier against water vapor, allowing the membranes to dry. The material from a hybridization bag should also work, provided it is thick enough.

It is important that, after color development, membrane pieces carrying DNA to be reamplified never dry, as otherwise reamplification by PCR may become impossible.

For documentation, scanning of the wrapped wet membranes has proved to yield the most satisfactory results.

Isolate and reamplify sample band

15. Cut out "differential" bands with a scalpel and transfer to microcentrifuge tubes each containing 20 µl TE buffer, pH 8.0. Make sure that membrane pieces do not become dry during this procedure. Using the scalpel tip, immediately submerge bands in the buffer. Rinse scalpel carefully before excising the next band.

    If excision is not intended to occur immediately, wet membranes can be stored 1 to 2 days at 4°C; however, during prolonged storage, wet membranes tend to become blotched. It is therefore advisable to dry membranes after at most one week. To avoid fading after drying, membranes should be kept dark (indefinitely) at room temperature.

16. For band reamplification, transfer half of the respective piece of membrane into a PCR tube containing 30 µl of the following mixture:

    4.0 µl buffer from the tube in which the band was stored
    3.0 µl 10× PCR buffer
    2.25 µl 20 mM $MgCl_2$
    0.6 µl 10 mM dNTPs
    13.85 µl H_2O
    3.0 µl 4 µM CP28
    3.0 µl 4 µM ML18
    0.3 µl 5 U/µl *Taq* DNA polymerase.

17. Amplify under the following conditions:

Initial step:	1 min	94°C (denaturation)
20 or 25 cycles:	20 sec	94°C (denaturation)
	20 sec	65°C (annealing)
	2 min	72°C (extension)
Final step:	indefinitely	10°C (hold).

    Amplification takes place for 20 cycles (strong bands) or 25 cycles (weak bands), respectively.

    Do not use biotinylated primers for band reamplification. 5′-modification of oligonucleotide primers will interfere with cloning.

18. Check products by agarose gel electrophoresis (*UNIT 2.5A*).

Clone products

19. Clone reamplification products as described in *UNIT 15.7* or by using one of the commercially available T-A cloning systems.

    In the authors' laboratory, 4 to 5 clones per band are usually sequenced. Depending on band intensity, all clones may be identical, or there may be more than one sort of insert. In the latter case, choose the most frequently occurring insert for further processing.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Blocking reagent, 1.5%

Prepare stock solution by suspending blocking reagent (Roche Molecular Biochemicals) to 10% (w/v) in maleic buffer, pH 7.5 (see recipe) and autoclaving. Store frozen up to 1 year at −20°C. Immediately before use, dilute 1.5 parts (v/v) of the 10% stock with 8.5 parts (v/v) of maleic buffer.

Maleic buffer, pH 7.5

100 mM maleic acid
150 mM NaCl
200 mM NaOH

Store indefinitely at room temperature

MboI-linker ML2025

Combine:
150 µl 100 pmol/µl ML20: 5′-TCA CAT GCT AAG TCT CGC GA-3′ (see *UNIT 2.11*)
150 µl 100 pmol/µl LM25: 5′-GAT CTC GCG AGA CTT AGC ATG TGA C-3′ (see *UNIT 2.11*)
55 µl 10× ligation buffer
195 µl H_2O.

Mix and place in a 90°C heating block. Shut off the heating block and let cool down slowly to room temperature. The linker (~0.5 µg/µl) is now ready for use and should be stored frozen up to 1 to 2 years at −20°C.

Alternatively, a thermocycler programmed to a low cooling rate (e.g., 0.02°C/sec) can be used as opposed to a heating block.

PCR buffer, 10×

670 mM Tris·Cl, pH 8.8 (*APPENDIX 2*)
170 mM $(NH_4)_2SO_4$
1% (v/v) Tween 20
Store up to 2 years at −20°C

PEG 8000, 50%

Add exactly 10 g of PEG 8000 (Promega) to 10 g water in a 50-ml conical tube (e.g., Becton Dickinson). Close the tube and attach to the rotor of a hybridization oven with the heat turned off. Rotate at room temperature 12 hr to overnight until all flakes are completely dissolved. Store up to 1 to 2 years at −20°C.

After thawing, shake vigorously until no more "schlieren" can be observed. Wait ~10 to 15 min until all air bubbles introduced by shaking have come to the surface before slowly and carefully withdrawing the desired volume.

It is important to adhere to the exact 1:1 weight ratio of PEG and water.

PEG 8000, 28%/$MgCl_2$, 3.6 mM

Carefully mix 5.6 ml 50% PEG 8000 (see recipe) with 3.68 ml water and 720 µl of 50 mM $MgCl_2$ (*APPENDIX 2*). Store up to 2 years at −20°C.

Reaction buffer

100 mM NaCl
5 mM Tris hydrochloride
90 mM Tris base
Store up to 1 year at room temperature

COMMENTARY

Background Information

Identification of differentially expressed genes is currently one of the most promising approaches toward understanding fundamental life processes. However, due to the high complexity of mRNA composition in a living cell, as well as the broad range of relative frequencies of particular transcripts and the fact that subtle changes in the expression level of a gene can have profound biological effects, performing a sensitive, reliable, and relatively complete comparative expression analysis has remained a nontrivial task up to the present.

Probably the first methods for isolation of differentially expressed genes that found widespread acceptance were the fingerprinting techniques of differential display (e.g., Liang and Pardee, 1992; see also *UNIT 25.B3*) and RNA arbitrarily primed PCR (Welsh et al., 1992). These methods relied on the generation of arbitrarily primed amplification products, each representing a particular transcript, which were radiolabeled and separated by polyacrylamide gel electrophoresis. Resulting band patterns originating from different samples were then compared. An indisputable strength of display technology, as opposed to subtractive hybridization experiments (*UNIT 25.B2*), is the option to directly compare any desired number of different samples with each other. Moreover, no prior knowledge about the RNA to be analyzed is required, rendering these methods suitable for analysis of RNA from any source. Nevertheless, in some hands, the application of these protocols was not always satisfactory (Debouck, 1995), due to insufficient reproducibility (Malhotra et al., 1998), a high rate of isolating false positive clones (Poirier et al., 1997), a biased representation favoring abundant transcripts (Ledakis et al., 1998), and contamination of workspaces through closed tube walls by volatile sulfur compounds (Trentmann, 1995). The use of longer primers (i.e., 20-mers; Zhao et al., 1995) improved reproducibility, but not other problems.

To address these issues, arbitrarily primed PCR was replaced by amplification of linker ligated restriction fragments (Fischer, 1995; Fischer et al., 1995; Prashar and Weissman, 1996). With this approach, it is possible to generate and display exactly one fragment per cDNA, thereby clearly increasing the sensitivity of the analysis. Spiking experiments demonstrated that, following the RMDD protocol as described above, an mRNA species at a relative concentration of 1:100,000 will usually be identifiable by a specific band. This holds true for the radioactive as well as for the nonradioactive version of the protocol—i.e., the authors could not detect any differences in the sensitivity of RMDD regardless of whether biotin or ^{33}P was used for labeling, which is due to the fact that sensitivity is not limited by the amount of amplification product used for display, but by a slight background smear which cannot be avoided when separating complex mixtures of PCR products by gel electrophoresis.

It is important to note that, due to the use of nonphosphorylated linkers, only one of the two linker strands is covalently attached to the cDNA restriction fragments upon ligation. The opposite linker strand is melted off during the initial denaturation step and can no longer serve as a primer binding site. Thus, amplification can take place only when extension of a nonlinker primer (i.e., the "downstream" primer which is essentially identical to the cDNA primer) has taken place, incorporating the reverse complement of the covalently attached linker strand. As a consequence, only cDNA 3′-ends are amplified to a detectable level, whereas "internal" cDNA fragments flanked by linkers at both ends remain unamplified.

Another problem that had to be solved was band identification. "Classic" protocols rely on cutting out invisible radioactive bands from dried gels after superimposing the gel and its corresponding autoradiogram (Liang and Pardee, 1992). In addition to the uncertainty of cutting invisible bands, which may easily lead to missing the desired band, tiny splinters of the radioactive gel, which becomes quite brittle after drying, might be inhaled. On the other hand, nonradioactive in-gel detection of DNA by silver staining turned out to lack sufficient sensitivity, and also significantly reduced the dynamic range of display patterns (A. Fischer, unpub. observ.). Attempts to bypass the physical fragment isolation step by defining fragment signatures and performing database searches after fluorescent gel display on an automatic DNA sequencer (A. Fischer, unpub. observ.; Shimkets et al., 1999; Sutcliffe et al., 2000) are hampered by the unpredictable influence of base composition on the electrophoretic mobility of a DNA strand, which introduces considerable inaccuracies when fragment sizes are to be determined, and are unsuitable for organisms less well characterized molecularly

Table 25B.4.1 Troubleshooting Guide for RMDD

Problem	Possible cause	Solution
Low amount of first-round PCR product	RNase contamination	Take care to use only RNase-free solutions. Make sure RNA is not contaminated by remaining traces of RNase. Check integrity of ribosomal bands after cDNA first-strand synthesis.
	RNA preparation contaminated by inhibitors of cDNA synthesis	Use only RNA that is as pure as possible. Usually, standard purification protocols (e.g., the "classic" guanidinium method, *UNIT 4.2*, or more modern, commercially available RNA purification columns), if not overloaded, yield RNA of sufficient purity. Should problems persist, in very tenacious cases purification of RNA by CsCl density gradient centrifugation (*UNIT 4.2*) might be considered.
	Incomplete PEG precipitation	Be sure to exactly balance the amounts of DNA solution and of PEG solution.
	Inefficient ligation	Check activity of ligase or use a fresh batch. Make sure linkers fit to the fragment ends generated by the employed restriction enzyme.
Agarose gel appearance of first-round PCR products obtained with identical primer combinations between samples	Very low amounts of template DNA lead to stochastical effects in early PCR cycles ("Monte Carlo effect"; Karrer et al., 1995)	See "low amount of PCR product"
Fuzzy bands on DBE membrane	Glass plates accumulated too much silane	Immerse glass plates for 1 hr in 0.5 M NaOH
	Edges of glass plates not exactly parallel	Make sure plates are carefully aligned immediately after pouring gel
Low signal intensity after color development	Biotin label of blotted DNA not sufficiently accessible	Use biotinylated PCR primers distinguished by a TEG spacer
	Insufficient amounts of second-round PCR primers	Check primer concentration. Since primers are used at limiting concentration, inaccuracies upon determination of concentration may hamper generation of sufficient PCR product.
White vertical stripes interrupt band pattern on membrane	Air bubbles accumulated at the lower edge of the gel	Degas running buffer for lower chamber by stirring 20 min under vacuum. Insert glass plates slightly inclined.
Band reamplification fails	UV fixation too strong	Apply ~$^1/_{10}$ the UV dose usually chosen for fixing DNA blots (recommended dose is 10,000 $\mu J/cm^2$)
	Membrane has become dry before reamplification	Keep wet membrane between two sheets of thick polyethylene wrap until bands are cut out. After cutting out bands, immediately submerge in buffer.
False positive clones (no regulation detectable)	Reamplification product contained more than one DNA species	Sequence more than one clone per band. If several inserts are identified, choose the most frequently occurring one.

than man or mouse. Attempts to use such an approach for analysis of rat RNA resulted in an unacceptably low hit rate (i.e., <10%) of correctly identified fragments (A. Fischer, unpub. observ.). Thus, although the RMDD technique could be very well performed using ^{32}P or ^{33}P as a label, the authors developed a protocol using nonradioactive detection of biotinylated amplification products. These are transferred to a membrane by use of direct blotting electrophoresis (DBE; Beck and Pohl, 1984); visible bands are rendered directly accessible by simply cutting them out of the stained membrane, eliminating one of the most common sources of false positives. Besides providing convenient access to "differential" bands, DBE proved superior in terms of resolution power, yielding nearly equally spaced bands in the range between 100 and 1000 base pairs.

After generating a library of cDNA-derived restriction fragments ligated to linkers, sufficient resolution and sensitivity of detection for the thousands of 3′-cDNA fragments generated has to be achieved. Towards this end, RMDD primers elongated at their 3′-ends are used for amplification, each only allowing the amplification of a defined subset of fragments. Using extensions of two nucleotides on each side, 16 linker primers and 12 reverse primers (the first extension nucleotide by definition cannot be a T) are synthesized. Thus, during the subsequent PCR step, the original set of fragments is divided into $16 \times 12 = 192$ subsets, which fits exactly in two 96-well PCR plates and renders the method suitable for automation. Each of these subsets then contains an estimated 50 to 100 fragments, which can be easily resolved by denaturing polyacrylamide electrophoresis.

Critical Parameters

During efforts to identify critical steps contributing to the robustness of the RMDD protocol, the amount of amplifiable material left after enzymatic processing of input RNA was identified as a major factor causing instability of band patterns, probably due to random fluctuations during early PCR cycles (Karrer et al., 1995). The authors' RMDD protocol was therefore optimized to minimize losses during sample preparation. Toward this end, an important step was replacing spin-column chromatography for removal of unincorporated linker molecules with size-selective polyethylene glycol precipitation, allowing almost 100% recovery of the desired DNA species and reduction of the protocol to as few steps as possible. In its current version, RMDD yields highly reproducible band patterns, independent of moderate variations of the amount of input material, in the range of at least down to 10 µg total RNA; however, it is essential that the analyzed RNA be of high purity. Otherwise, due to inhibition of enzymatic steps, the amount of linker-ligated template fragments effectively entering amplification might become too low to guarantee stable PCR results.

One should also be aware that RMDD analysis only covers those transcripts that carry a recognition site for the restriction enzyme used in an "amplifiable" distance from the poly(A) tail. For a more detailed discussion of this issue, see Strategic Planning.

Troubleshooting

For solutions to problems that may arise during these protocols, see Table 25.B4.1.

Anticipated Results

After corresponding PCRs (distinguished by identical primer extensions) from different RNA samples are run side by side on the gel, resulting band patterns are visually compared. A typical RMDD pattern shows, in each lane, bands of different sizes and intensities, each representing one particular cDNA. Patterns obtained from similar samples should very closely resemble each other, with only very few (if any) differences. Within these patterns, band intensities correlate with the original relative frequencies of the template cDNAs. This is due to the fact that in complex PCR reactions (i.e., with more than one amplification product) entry into plateau phase of amplification freezes the different amounts of synthesized products (McClelland and Welsh, 1994); therefore, if a particular cDNA is present at different amounts in the two samples, the resulting bands will show different intensities on the RMDD membrane. Differences in expression levels at least down to 2-fold will be detectable. In one instance, a band that was shown by quantitative PCR to represent a gene down-regulated 1.4-fold was isolated in the authors' laboratory. This is especially significant as the "gold standard" in transcription profiling is usually set at 2-fold up- or down-regulation; therefore, the fact that RMDD allows isolation of transcripts regulated to a lower degree, which still can be of the highest biological relevance (e.g., gradients in developmental biology), clearly contributes to its usefulness.

Time Considerations

When starting with up to six samples of precipitated RNA, the protocol, including second-round amplification with a subset of all primer combinations, can be performed within two days, including an overnight ligation step. The remaining set of second-round amplifications can be done at a rate of four to six 96-well plates per day and person. Alternatively, employment of a robotic pipetting station might be considered. Choosing the DBE variant, two membranes per day per DBE machine can be prepared, each providing space for 30 reactions. It should be noted that buffer capacity allows for using each DBE gel twice, provided that the second run starts immediately after the first run without idle electrophoresis in between. One person can then operate three to four machines per day and produce 6 to 8 membranes. In such a medium-scale setup, gels are prepared in the evening, and, with edges carefully wrapped in plastic wrap with some wetted pieces of paper towel enclosed, allowed to polymerize overnight. In the morning, gels are mounted and electrophoresis is started. During electrophoresis, the membranes of the day before are developed, the glass plates of the previous runs cleaned, and the gels for the next day are prepared.

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AFLP-Based Transcript Profiling

In recent years, several techniques have been developed to analyze the transcriptome—i.e., the entirety of transcripts present in a cell, tissue, or organ. These procedures include methods based on hybridization to microarrays of known expressed sequence tag (EST)-sequences (Schena et al., 1995; De Risi et al., 1997), sequence-based approaches like SAGE (Velculescu et al., 1995; *UNIT 25B.6*) and random EST sequencing (Adams et al., 1991), and protocols based on display of cDNA fragment patterns on high-resolution gels (Liang and Pardee, 1992; *UNITS 25B.3 & 25B.4*). In the last category is transcript profiling based on amplified fragment length polymorphism (AFLP)-fingerprinting of double-stranded cDNA (Zabeau and Vos, 1993; Vos et al., 1995; Bachem et al., 1996). The protocol, illustrated in Figures 25B.5.1 and 25B.5.2, includes the following steps: (1) the isolation of poly(A)$^+$ RNA from total RNA (*UNIT 4.2*), (2) the synthesis of a double-stranded (ds) cDNA library, (3) the preparation of template fragments by digestion of the cDNA library with a combination of two restriction enzymes and the ligation of adapters to the fragment ends, (4) the selective amplification of specific subsets of fragments, and (5) the electrophoretic analysis of these amplification products on standard denaturing polyacrylamide gels. The protocol given in this unit describes all steps in the procedure, except the isolation of total RNA; however, any of the presently used methods is acceptable (e.g., *UNIT 4.2*). The restriction enzyme combination (EC) used in this protocol is *Taq*I-*Mse*I. This EC will target the majority of the mRNAs, and both *Mse*I and *Taq*I are reliable and inexpensive. Other combinations of two 4-base cutters may also work well

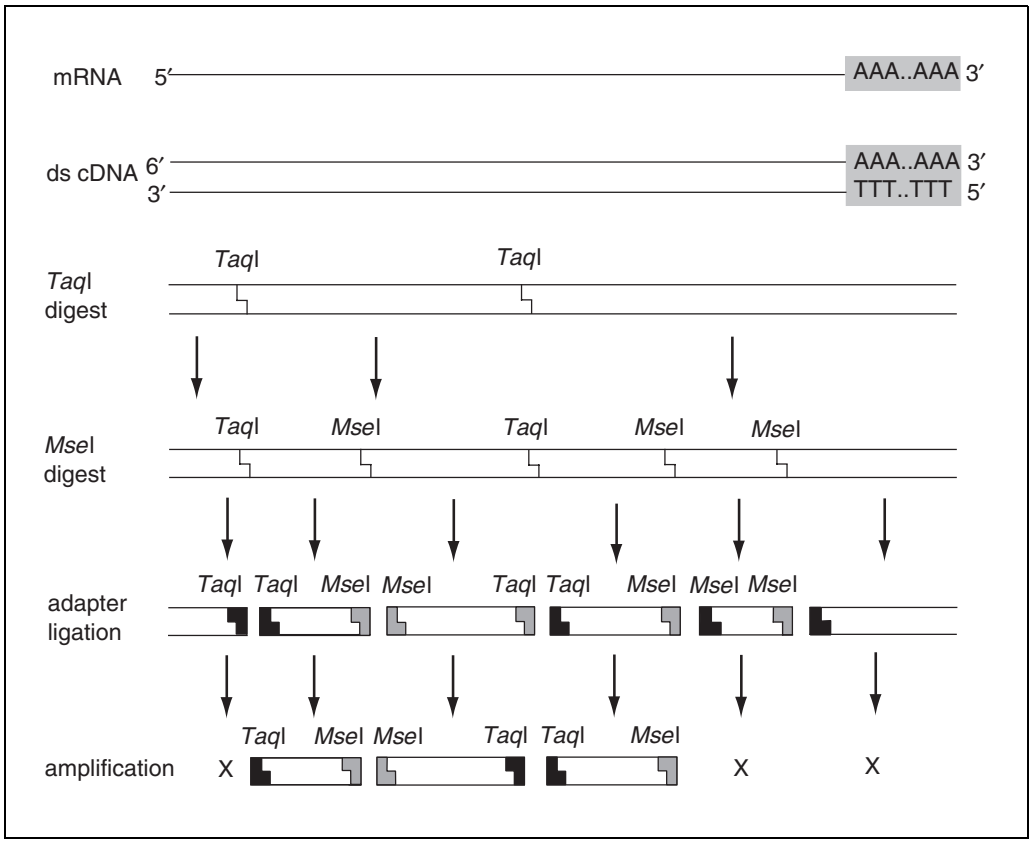

Figure 25B.5.1 Principle of the AFLP-based transcript profiling technique. The poly(A)$^+$ RNA is indicated at the top with the poly(A) tail at the 3′ end. The ds cDNA is shown as a double line; restriction enzyme sites with 5′ overhangs are indicated. The ds *Taq*I and *Mse*I adapters are depicted as small black and gray boxes respectively, attached to the protruding ends of the restriction fragments. At the bottom the "X's" illustrate the poor amplification of the *Mse*I-*Mse*I fragments.

Contributed by Pieter Vos and Patrick Stanssens

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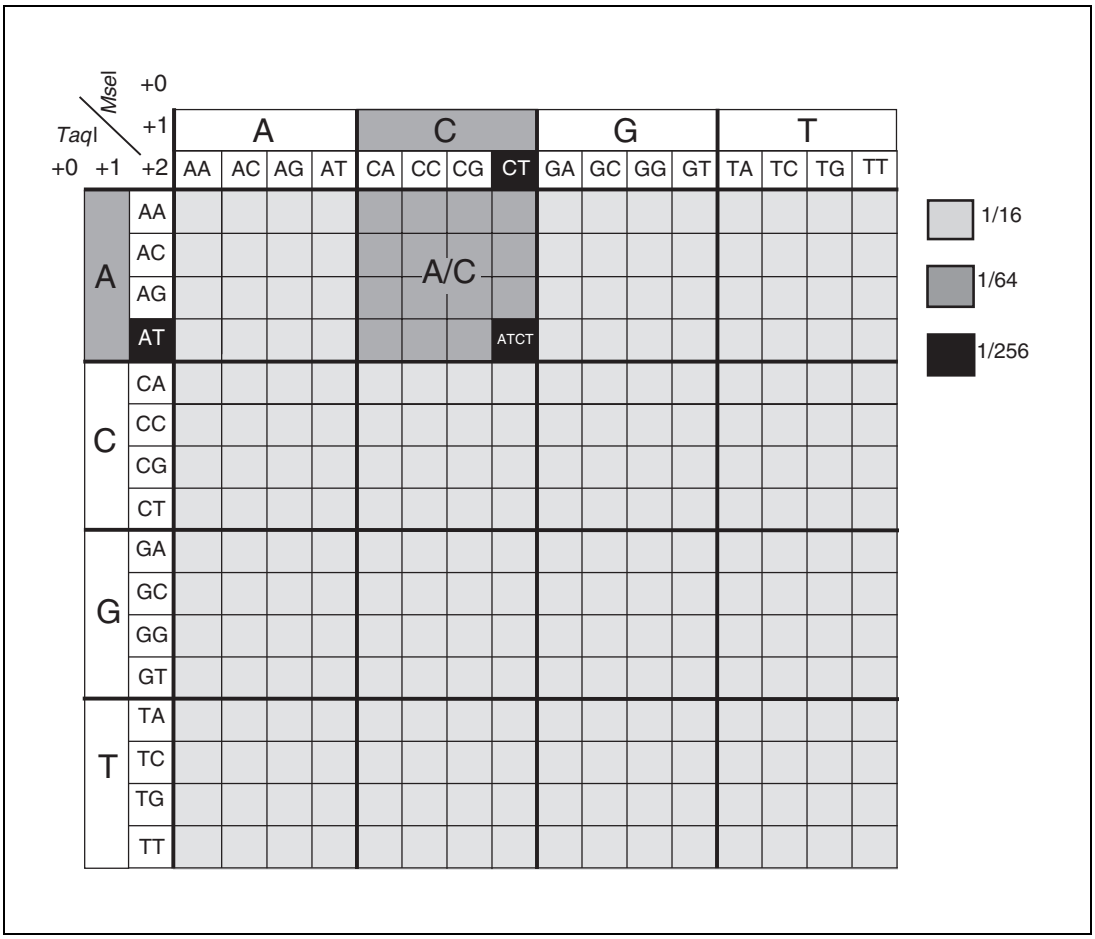

Figure 25B.5.2 Illustration of the selective amplification principle. The smallest squares indicate subsets of the transcript fragment population amplified with four selective nucleotides, two for *Taq*I and two for *Mse*I, and exemplified by the black square amplified with *Taq*I-AT and *Mse*I-CT. The 16 larger squares, composed of 16 of the smallest squares, indicate the transcript fragment subsets amplified using two selective nucleotides, exemplified by the dark gray square of *Taq*I-A and *Mse*I-C. The total transcript fragment population is depicted by the full square, composed of 256 of the smallest squares.

but require the use of adaptors and primers that match the recognition sequences of the corresponding enzymes (see Reagents and Solutions). Restriction enzymes that cut less frequently in the cDNA are not advised since these enzymes target only a small subset of the mRNAs.

To generate specific subsets of fragments, three PCR steps are used, which minimizes mismatch amplification. When all combinations of PCR primers are used at each step, as prescribed in the protocol, this generates an expression profile consisting of 256 "fingerprints" (Fig. 25B.5.2). (One can modify the protocol to use only certain primer combinations, but this will yield fewer fingerprints and less information.) The first PCR step entails no selective nucleotides on each primer (i.e., nonselective preamplification +0/+0). The second step entails one selective nucleotide at each primer (selective preamplification +1/+1; 16 combinations). The third step entails two selective nucleotides at each primer (selective amplification +2/+2; 256 combinations).

NOTE: All solutions and materials coming into contact with RNA must be RNase free, and proper techniques should be used accordingly (see *APPENDIX 2*).

NOTE: AFLP is a registered trademark of Keygene N.V. and is protected by patents and patent applications of Keygene N.V.

Materials

Total RNA (*UNIT 4.2* or equivalent)
5′-biotinylated oligo-dT_{25} (5-biotin-dT_{25})
1× and 2× binding buffer (see recipe)
H_2O: Milli-Q purified (i.e., water deionized by passage through a five-stage Milli-Q Plus system; Millipore) or double-distilled
Streptavidin-coated magnetic beads (Dynal)
Wash buffer (see recipe)
2 mM EDTA, pH 7.5
5× first-strand buffer (see recipe)
5× second-strand buffer (see recipe)
0.1 M DTT (*APPENDIX 2*)
5 and 10 mM (each) mixture of all 4 dNTPs (Pharmacia or *UNIT 3.4*)
SuperScript II (Life Technologies)
E. coli DNA ligase (Life Technologies)
E. coli DNA polymerase I (Pharmacia Biotech)
RNase H (Pharmacia Biotech)
2× and 1× STEX (see recipe)
10 mM Tris·Cl, pH 8.0/0.1 mM EDTA (*APPENDIX 2*)
*Taq*I restriction endonuclease (New England Biolabs; *UNIT 3.1*)
5× RL buffer (see recipe)
*Mse*I restriction endonuclease (New England Biolabs; *UNIT 3.1*)
50 pmol/µl *Taq*I adapter top and bottom strands (see recipe for oligonucleotides and double-stranded adapters)
50 pmol/µl *Mse*I adapter top and bottom strands (see recipe for oligonucleotides and double-stranded adapters)
10 mM ATP (Pharmacia)
T4 DNA ligase (Pharmacia)
8 pmol/µl AFLP + 0 (nonselective) primers (see recipe for oligonucleotides and double-stranded adapters): *Taq*I + 0 and *Mse*I + 0 primers
10× PCR buffer (see recipe)
AmpliTaq DNA polymerase (Perkin-Elmer; *UNIT 3.5*)
10 µCi/µl (~2000 Ci/mmol) [^{33}P-γ]ATP (Amersham)
10× T4 polynucleotide kinase buffer (see recipe)
T4 polynucleotide kinase (Pharmacia; *UNIT 3.4*)
8 pmol/µl AFLP +1 and + 2 (selective) primers (see recipe for oligonucleotides and double-stranded adapters): *Taq*I + 1 and + 2 and *Mse*I + 1 and + 2 primers
AmpliTaq-Gold polymerase (Perkin-Elmer)
Loading dye (see recipe)
Repel silane (Pharmacia)
Bind silane solution, fresh: Combine 30 µl bind silane (Pharmacia Biotech) and 30 µl glacial acetic acid in 10 ml ethanol
4.5% denaturing polyacrylamide gels (see recipe)
1× TBE (see recipe)
Molecular weight standard (e.g., SequaMark 10-base ladder; Research Genetics; optional)
10% acetic acid

Microcentrifuge tubes, RNase free
Magnetic plate chamber (MPC; Dynal)
PE-9600 thermal cycler (Perkin Elmer) and PCR microtiter plate

Sequencing gel system (e.g., BioRad 38 × 50 × 0.04–cm SequiGen sequencing gel system)

PhosphorImager (Fujix BAS 2000, Molecular Dynamics STORM 824)

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5A*), analysis by denaturing polyacrylamide gel electrophoresis (*UNIT 2.12*), and detection of DNA by autoradiography or phosphor imaging (*APPENDIX 3A*)

NOTE: Suppliers and brands are generally not very critical, however in case of problems it is advised to use the suggested suppliers for at least the reverse transcriptase (SuperScript II) and *Taq* polymerases (AmpliTaq and AmpliTaq-Gold).

NOTE: When preparing AFLP amplifications, it is advisable to work with mixes of reagents as much as possible. Working with mixes facilitates assembly and is also important for the reliability and reproducibility of the reactions. In practice, the assembly of the mixes depends on the experiment—i.e., which components remain constant in a series of reactions: the template-DNA or the primer combinations (e.g., one sample with many primer combinations, many samples with one primer combination).

Isolate poly(A)⁺ RNA

1. Combine 200 µg total RNA, 600 ng 5′-biotinylated oligo-dT_{25} (5-biotin-dT_{25}), and 300 µl 2× binding buffer in an RNase-free microcentrifuge tube. Adjust the volume to 600 µl with water. Incubate 5 min at 70°C, followed by 15 to 20 min at room temperature.

   Sufficient poly(A)⁺ RNA to perform the subsequent steps (i.e., cDNA synthesis and template preparation in duplicate) is yielded from 200 µg total RNA.

2. Wash 150 µl streptavidin-coated magnetic beads with 0.5 ml of 1× binding buffer (see step 4 below for technique or use microcentrifuge). Resuspend the beads in 50 µl of the same buffer.

   Mix the magnetic beads solution well before use to obtain a homogeneous suspension. Do not let the magnetic beads dry for a long period of time, as drying may lower their capacity (see Dynal, 1995).

3. Add these prewashed beads to the RNA-containing mixture (step 1) and incubate 30 min at room temperature with gentle agitation.

4. Place the microcentrifuge tube in the magnetic plate chamber (MPC) for ~30 sec and then remove as much of the supernatant as possible without disturbing the beads. Remove the tube from the MPC, add 0.5 ml wash buffer, and mix thoroughly. Repeat two more times, removing the supernatant after the final wash.

   Do not allow the beads to dry out.

5. Elute poly(A)⁺ RNA by resuspending the beads in 20 µl of 2 mM EDTA and incubating 5 min at 70°C. Collect the beads with the MPC as in step 4 and transfer the supernatant to a new RNase-free microcentrifuge tube as quickly as possible without transferring any beads. Repeat once to obtain ~40 µl of poly(A)⁺ RNA solution.

   For long-term storage, add 0.1 vol 2 M sodium acetate, pH 5.5 and mix. Add 3 vol 100% ethanol and store indefinitely at −20°C (UNIT 2.1A). To recover, microcentrifuge 5 min at maximum speed, remove supernatant, dry in a rotary evaporator, and resuspend in the original volume of double-distilled water or buffer.

6. Check the yield (on average ~2 µg) and quality of the isolated poly(A)⁺ RNA by performing agarose gel electrophoresis alongside molecular weight markers (*UNIT*

2.5A) using 5 µl of the poly(A)$^+$ RNA solution, which should appear as a faint smear from ~10 kb down (i.e., lower molecular weight) with trace rRNA bands.

It is not necessary to eliminate the rRNA contamination by extracting the mRNA from the eluate a second time.

Synthesize the ds cDNA

7. For first-strand cDNA synthesis, combine the following:

   10 µl poly(A)$^+$ RNA (~0.5 µg)
   0.5 µl 700 ng/µl 5-biotin-dT_{25} (reverse transcription primer)
   2 µl H_2O
   4 µl of 5× first-strand buffer
   2 µl 0.1 M DTT
   1 µl 10 mM dNTPs
   0.5 µl of 200 U/µl SuperScript II (add last).

   Incubate 2 hr at 42°C.

8. For second-strand synthesis, combine the following:

   20 µl first-strand cDNA synthesis mixture (from step 7)
   16 µl 5× second-strand buffer
   1.5 µl 10 mM dNTPs
   3 µl 0.1 M DTT
   7.5 U *E. coli* DNA ligase
   25 U *E. coli* DNA polymerase I
   0.8 U RNase H
   H_2O to 80 µl.

   Incubate 1 hr at 12°C followed by 1 hr at 22°C.

   The quality and yield of the resulting ds cDNA can be checked by agarose gel electrophoresis (UNIT 2.5A).

9. Wash 25 µl streptavidin-coated beads with 100 µl of 2× STEX (see step 4 for technique). Resuspend in 80 µl of 2× STEX.

10. Add the bead suspension to the cDNA mixture and incubate 30 min at room temperature with gentle agitation.

    Purification of a large number of samples using beads can be performed in 96-well format. Incubation at room temperature is done in 96-well plates with caps. Subsequently, samples are transferred to fresh microtiter plates.

11. Collect beads with the MPC (step 4), wash once with 100 µl of 1× STEX, and transfer to a fresh microcentrifuge tube. Wash twice more with 1× STEX and resuspend final bead pellet in 50 µl H_2O or 10 mM Tris·Cl, pH 8.0/0.1 mM EDTA.

    Generally, 250 to 500 ng ds cDNA will be obtained from the 500 ng of input (single-stranded) poly(A)$^+$ RNA.

    10 mM Tris·Cl, pH 8.0/0.1 mM EDTA is also known as $T_{10}E_{0.1}$ buffer and has a lower EDTA concentration than the TE buffer described in APPENDIX 2 of this manual.

    The ds cDNA is attached to the beads and is taken into subsequent steps while attached to the beads.

Prepare the AFLP cDNA template fragments using TaqI and MseI

12. Mix the following:

    20 µl cDNA preparation (generally 100 to 200 ng cDNA)
    10 U *Taq*I restriction endonuclease
    8 µl 5× RL buffer
    Adjust volume to 40 µl with H_2O.

    Incubate 1 hr at 65°C.

13. Add the following:

    10 U *Mse*I restriction endonuclease enzyme
    2 µl 5× RL buffer
    Adjust the volume to 50 µl with H_2O.

    Incubate 1 hr at 37°C.

14. Prepare the *Taq*I adapter by combining 8.5 µg (1500 pmol) top and 8 µg (1500 pmol) bottom strands. Adjust volume to 30 µl with water.

    This results in a solution of 50 pmol/µl of double-stranded TaqI-adapter.

15. Prepare the *Mse*I adapter by combining 8.0 µg (1500 pmol) of the top strand and 8.0 µg (1500 pmol) of the bottom strand. Adjust volume to 30 µl with water.

    This results in a solution of 50 pmol/µl of ds MseI adapter.

    The TaqI and MseI adapters both have double-stranded parts of 14 base pairs; it appears unnecessary to perform a specific denaturation-renaturation procedure to anneal the two strands of the adapters. Note that the base-pair adjacent to the restriction site overhang is such that the recognition site is not restored upon ligation (see Background Information). Absence of 5′-phosphates prevents self-ligation of adapters.

16. To the cDNA fragments digested with *Taq*I and *Mse*I (steps 12 and 13), add 1 µl of each adapter (50 pmol each; steps 14 and 15), 1 µl of 10 mM ATP, 2 µl of 5× RL-buffer, 1 U of T4 DNA ligase, and 10 µl water. Incubate 2 hr at 37°C.

    The cDNA is incubated for 2 hr with restriction enzymes (steps 12 and 13) followed by an additional incubation of 2 hr in the presence of DNA ligase. It is not advisable to perform the restriction digestion and ligation simultaneously. This may affect the efficiency of the DNA restriction. Longer incubation times are also not recommended, because this may affect the quality of the transcript fingerprints. After digestion and ligation of adapters, the cDNA is stored at −20°C or immediately used for the subsequent steps.

Perform nonselective preamplification of the template fragments

17. Dilute a small aliquot (2 to 5 µl) of the template mixture (step 16) 10-fold with Tris·Cl, pH 8.0/0.1 mM EDTA. Prepare the following preamplification reactions:

    5.0 µl 1:10 diluted template mixture
    1.5 µl 8 pmol/µl each AFLP + 0 (nonselective) primer
    2.0 µl 5 mM dNTPs (0.2 mM final concentration of each dNTP)
    5 µl 10× PCR buffer
    1 U AmpliTaq DNA polymerase
    Adjust volume to 50 µl with H_2O.

18. Amplify using the following temperature cycle profile on a PE-9600 thermal cycler:

20 cycles:	30 sec	94°C	(denaturation)
	60 sec	56°C	(annealing)
	60 sec	72°C	(extension).

The purpose of the nonselective preamplification reaction is to generate more starting material for the subsequent selective AFLP reactions. This is one of the advantages of the AFLP technique. Once the template-DNA is made, new starting material for selective amplifications can always be made by nonselective amplification of the template DNA, and hence, new RNA isolation will never have to be done again.

Note that the adapter strands are not phosphorylated and that, therefore, the strand which represents the primer-target is not ligated to the template DNA. Thus, a "hot-start" should never be performed (UNIT 15.1); however, during the initial heating step, Taq polymerase should elongate the staggered ends of the template replacing the adapter strands.

19. Check the preamplification by running 10 µl of the reaction mixture on an agarose gel alongside molecular weight markers (*UNIT 2.5A*), which should give a visible smear of products in the size range of 50 to 500 base pairs.

Perform selective preamplification reactions using TaqI+1 and Mse+1 primers

20. Dilute 2 µl nonselective preamplified cDNA fragments (i.e., +0/+0) 1:500 in Tris·Cl, pH 8.0/0.1 mM EDTA.

21. Prepare selective preamplification (+1/+1) reactions in a microtiter plate for a PE-9600 thermocycler in the following way:

    a. Dispense 5 µl of 1:500 nonselective preamplification cDNA fragments into each well of the first two columns (1 and 2) of the microtiter plate.

    b. Dispense 1.5 µl of 8 pmol/µl *Taq*I+A primer into each of wells A1 to D1, 1.5 µl of 8 pmol/µl *Taq*I+C primer into each of wells E1 to H1, 1.5 µl of 8 pmol/µl *Taq*I+G primer into each of wells A2 to D2, and 1.5 µl of 8 pmol/µl *Taq*I+T primer into each of wells E2 to H2.

    c. Dispense 1.5 µl of 8 pmol/µl *Mse*I+A primer into each of wells A1, A2, E1, and E2; 1.5 µl of 8 pmol/µl *Mse*I+C primer into each of wells B1, B2, F1, and F2; 1.65 µl of 8 pmol/µl *Mse*I+G primer into wells C1, C2, G1, and G2; and 1.4 µl of 8 pmol/µl *Mse*I+T primer into each of wells D1, D2, H1, and H2.

    d. Prepare dNTP/polymerase mix by combining 32 µl 5 mM dNTPs, 80 µl of 10× PCR buffer, 16 U AmpliTaq-Gold polymerase, and adjust the volume to 672 µl with water.

    e. Dispense 42 µl dNTP/polymerase mix into each well of the first two columns.

    The procedure above can be adjusted when more samples are processed at the same time (i.e., 3 samples occupy 48 wells of the microtiter plate and three times more TaqI+1 primers, MseI+1 primers, and dNTP/polymerase mix will be needed).

    An individual reaction can be prepared by combining 5 µl of 1:500 nonselective preamplification product, 1.5 µl of 8 pmol/µl TaqI+1 primer (12 pmol), 1.5 µl of 8 pmol/µl MseI+1 primer (12 pmol), 2 µl of 5 mM dNTP, 5 µl 10× PCR buffer, and 1 U AmpliTaq-Gold polymerase. The volume is adjusted to 50 µl with water.

22. Perform AFLP amplification with the following "touch-down" temperature cycle program:

13 cycles:	30 sec	94°C	(denaturation)
	30 sec	65°–0.7°C/cycle	(annealing)
	60 sec	72°C	(extension)
23 cycles:	30 sec	94°C	(denaturation)
	30 sec	56°C	(annealing)
	60 sec	72°C	(extension).

The initial annealing step is performed at 65°C, decreasing by 0.7°C each cycle.

A stepwise amplification procedure is used to minimize mismatch amplification. A single additional selective nucleotide (one on each primer) is added per selective AFLP amplification. The most useful expression profiles consist of the 256 fingerprints obtained with all combinations of the TaqI+2 and MseI+2 primers. This implicates a series of 3 consecutive PCRs, the first with no selective nucleotides (nonselective preamplification +0/+0), the second with one selective nucleotide at both the TaqI and MseI primer (selective preamplification +1/+1), and the third with two selective nucleotides at each primer (final selective amplification +2/+2).

Similar to the nonselective preamplification, it is advisable to check 10 µl of the reaction mixtures on an agarose gel.

Label selective TaqI + 2 primers

23. Prepare the following phosphorylation reaction mixture:

    2.0 µl 10 µCi/µl (~2000 Ci/mmol) [^{33}P-γ]ATP
    1.0 µl 10× T4 polynucleotide kinase buffer
    4 U T4 polynucleotide kinase
    Adjust volume to 8 µl with water.

24. To phosphorylate 16 pmol selective *Taq*I+2 primer (the amount required for 20 AFLP reactions; i.e., the amount required to perform all 16 +2/+2 reactions for a given *Taq*I+2 primer in the complete set of 256 primer combinations), combine 2.0 µl of 8 pmol/µl selective primer (+2) and 8.0 µl phosphorylation reaction mix (step 23), yielding labeled primer at a concentration of 12.6 pmol/µl and a final volume of 10.0 µl. Incubate 60 min at 37°C, followed by 10 min at 70°C to inactivate the kinase.

    ^{33}P-labeled primers are preferred because they give a better resolution of the PCR products on polyacrylamide gels. Also, the reaction products are less prone to degradation due to autoradiolysis.

    Only the TaqI primers should be labeled. Labeling both the TaqI and MseI primers causes each of the two strands of the AFLP fragments to be visualized on the gels, often causing "doublets" when these two strands migrate differently on the gel.

Perform selective AFLP amplification using labeled TaqI + 2 and MseI + 2 primers

25. Dilute 2 µl of each selective preamplification product (+1/+1; step 22) 500-fold with Tris·Cl, pH 8.0/0.1 M EDTA. Prepare selective amplification (+2/+2) reactions in a microtiter plate for a PE-9600 thermocycler in the following way:

    a. Dispense 2 µl of 1:500 preamplification mixture *Taq*I+A/*Mse*I+C in the first two columns of the microtiter plate.

    b. Dispense 0.5 µl labeled *Taq*I+AA primer into each of wells A1 to D1; 0.5 µl labeled *Taq*I+AC primer into each of wells E1 to H1; 0.5 µl labeled *Taq*I+AG primer into each of wells A2 to D2, and 0.5 µl labeled *Taq*I+AT primer into each of wells E2 to H2.

    c. Dispense 0.6 µl unlabeled *Mse*I+CA primer into each of wells A1, A2, E1, and E2; 0.6 µl unlabeled *Mse*I+CC primer into each of wells B1, B2, F1, and F2; 0.6 µl unlabeled *Mse*I+CA primer into each of wells A1, A2, E1, and E2; 0.6 µl unlabeled *Mse*I+CG primer into each of wells C1, C2, G1, and G2; and 0.6 µl unlabeled *Mse*I+CT primer into each of wells D1, D2, H1, and H2.

    d. Prepare dNTP/polymerase mixture by combining 12.8 µl of 5 mM dNTPs, 32 µl of 10× PCR buffer, 6.4 U AmpliTaq-Gold polymerase, and adjusting the volume to 270.4 µl.

    e. Dispense 16.9 µl dNTP/polymerase mixture in the first two columns of the microtiter plate.

An individual reaction can be prepared by combining 2 µl of 1:500 selective preamplification reaction product, 0.5 µl of 1.6 pmol/µl (5 ng) labeled selective TaqI+2 primer, 0.6 µl of 8 pmol/µl (30 ng) unlabeled selective MseI+2 primer, 0.8 µl of 5 mM dNTPs, 2.0 µl of 10× PCR buffer, and 0.4 U AmpliTaq-Gold polymerase. The volume is adjusted to 20 µl with water.

26. Amplify the material using the "touch-down" PCR program specified in step 22.

Generally, a number of AFLP reactions will be performed in parallel and the indicated quantities of the reaction mixes should be adjusted accordingly.

Analyze amplification products by standard PAGE

27. Mix the AFLP reactions with an equal volume (20 µl) of loading dye. Denature the AFLP reaction products by heating at 90°C for 3 min and then quickly cooling on ice.

CAUTION: *Formamide is harmful—perform this step under a fume hood.*

28. Treat the back plate of the sequencing gel system with 2 ml repel silane, and the front plate with 10 ml bind silane solution. Prepare 4.5% denaturing polyacrylamide gels (~100 ml).

The authors use the BioRad SequiGen sequencing gel system (38 × 50 × 0.04–cm), for which the parameters given in this protocol are optimized; however, other sequencing gel systems should also work well.

29. Using 1× TBE as the running buffer, prerun gels 0.5 hr just before loading the samples under appropriate conditions to heat the gel to ~55°C (e.g., 110-W limit for the BioRad system). Use a gel thermometer to monitor temperature.

Maintaining this temperature throughout the electrophoresis is crucial for good quality fingerprints.

30. Load either 3 µl (for 48-lane gels) or 1.5 µl (for 96-lane gels) of sample into each well and analyze at ~55°C. Include a molecular weight standard (e.g., SequaMark 10-base ladder) if desired.

31. After electrophoresis, disassemble the gel cassette. Fix the gel, which will stick to the front glass plate because of the silane treatments, by soaking in 10% acetic acid for 30 min. Rinse thoroughly with water and dry 10 to 20 hr at room temperature in a fume hood, or for a shorter time period at an elevated temperature (e.g., using an incubator).

CAUTION: *Radioactive materials require special handling. See APPENDIX 1F and the institutional Radiation Safety Office for guidelines concerning proper handling and disposal.*

Gel is dry when it is no longer "sticky."

32. Visualize gel-fractionated cDNA AFLP fragments by autoradiography or using a phosphorimager (*APPENDIX 3A*).

Exposure times are reduced at least 2.5-fold using phosphorimaging technology.

REAGENTS AND SOLUTIONS

Use Milli-Q purified or double-distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Binding buffer, 2×, 1×

20 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
150 mM LiCl
1 mM EDTA (*APPENDIX 2*)
Store up to 6 months at room temperature
Dilute to 1× with Milli-Q-purified or double-distilled H_2O

Denaturing polyacrylamide gel, 4.5%

Prepare 4.5% (v/v) Sequagel ready-for-use gel mix (19:1 acrylamide/methylene bisacryl; National Diagnostics) in 7.5 M urea (Life Technologies)/0.5× TBE (see recipe) at a total volume of ~100 ml. Add 500 µl of 10% ammonium persulfate (APS), freshly made just before use, and 100 µl of TEMED (*N*,*N*,*N*',*N*'-tetramethylethylenediamine) immediately before casting the gel. Cast the gel according to the instructions of the gel system manufacturer, using either two 24-well (for 48-lane gels) or 48-well (for 96-lane gels) sharkstooth combs to create the gel slots.

These gels are essentially normal sequencing gels (Vos and Kuiper, 1998; UNIT 7.6), with the exception that a lower percentage of polyacrylamide is used. Ready made solutions should also work well.

First-strand buffer, 5×

250 mM Tris·Cl pH 8.3 (*APPENDIX 2*)
15 mM $MgCl_2$
375 mM KCl
Store up to 6 months at −20°C

Oligonucleotides and double-stranded adapters

Adapters:
*Taq*I adapter top strand: 5′-CTCGTAGACTGCGTACA-3′
*Taq*I adapter bottom strand: 3′-CATCTGACGCATGTGC-5′
*Mse*I adapter top strand: 5′-GACGATGAGTCCTGAG-3′
*Mse*I adapter bottom strand: 3′-GCTACTCAGGACTCAT-5′

Nonselective primers (AFLP + 0):
*Taq*I + 0 primer: 5′-CTCGTAGACTGCGTACACGA-3′
*Mse*I + 0 primer: 5′-GACGATGAGTCCTGAGTAA-3′

Selective primers (AFLP +1 and +2):
*Taq*I + 1 primer: 5′-GTAGACTGCGTACACGAN-3′
TaqI + 2 primer: 5′-GTAGACTGCGTACACGANN-3′
*Mse*I + 1 primer: 5′-GATGAGTCCTGAGTAAN-3′
*Mse*I + 2 primer: 5′-GATGAGTCCTGAGTAANN-3′

N is any nucleotide; therefore, there are a total of 1 "+ 0," 4 "+ 1," and 16 "+2" primers for each restriction endonuclease.

Loading dye

98% formamide, deionized and filtered (Merck)
10 mM EDTA, pH 8.0 (*APPENDIX 2*)
5 mM spermidine·3HCl (Sigma)
Trace amounts (i.e., ~0.5 mg/ml) of bromphenol blue and xylene cyanol
Store in small (500 µl) aliquots up to 6 months at −20°C.

PCR buffer, 10×

100 mM Tris·Cl, pH 8.3 (*APPENDIX 2*)
15 mM $MgCl_2$
500 mM KCl
Store up to 6 months at room temperature

RL buffer, 5×

50 mM Tris acetate, pH 7.5
50 mM magnesium acetate
250 mM potassium acetate
25 mM DTT
Store in small aliquots (up to 500 µl) and store up to 6 months at −20°C

Second-strand buffer, 5×

100 mM Tris·Cl, pH 7.0 (*APPENDIX 2*)
20 mM $MgCl_2$
450 mM KCl
750 µM NAD^+
50 mM $(NH_4)_2SO_4$
Store in small aliquots up to 6 months at −20°C

STEX, 2×, 1×

20 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
2000 mM NaCl (*APPENDIX 2*)
2 mM EDTA (*APPENDIX 2*)
0.2 % (v/v) Triton X-100
Store up to 6 months at room temperature
Dilute to 1× with Milli-Q-purified or double-distilled H_2O

T4 polynucleotide kinase buffer, 10×

250 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
100 mM $MgCl_2$
50 mM DTT
Make small aliquots and store up to 6 months at −20°C

TBE, 1×

Prepare a 10× stock:
1 M Tris base
1 M boric acid
20 mM EDTA, pH 8.3
Store up to 6 months at room temperature
Dilute to 1× with water

Wash buffer

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
150 mM LiCl
1 mM EDTA
Store up to 6 months at room temperature

COMMENTARY

Background Information

At present, a variety of technologies are available for high-throughput analysis of mRNA populations in cells, tissues, and organs. These can be divided into three major classes: (1) methods based on hybridization of labeled cDNA to transcript sequences on microarrays (Schena et al., 1995; De Risi et al., 1997), (2) methods based on high-throughput sequencing of small identifier ("signature") sequences corresponding to specific transcripts (*UNIT 25B.6*; Velculescu et al., 1995; Brenner et al., 2000), and (3) methods based on display of cDNA fragment patterns on high-resolution gels such as AFLP (*UNITS 25B.3 & 25B.4*; Liang and Pardee, 1992; the current unit).

Hybridization to microarrays of known transcript sequences is an attractive method for high-throughput transcript analysis (Schena et al., 1995; De Risi et al., 1997). The amount of data that can be obtained with this technology cannot be matched easily by any other presently known transcript analysis method. The fast growing number of gene and whole genome sequences creates a valuable resource for probe design for microarrays. One of the most attractive applications of the technology to date is the comparative analysis of gene expression between two samples for which the cDNA is differentially labeled (Welsh et al., 2001). Cross hybridization may pose a problem using microarrays, primarily because gene families are quite predominant in higher organisms; however, the use of multiple oligonucleotide probes of individual genes alleviates this problem, enabling the design of highly discriminative oligonucleotide sets (Wodicka et al., 1997).

A second category of transcript analysis technologies is represented by the SAGE technology (Serial Analysis of Gene Expression) first described by Velculescu et al. (1995; *UNIT 25B.6*) and the Massive Parallel Signature Sequencing (MPSS) technology first described by Brenner et al. (2000). These technologies generate small identifier or signature sequences specific for each transcript in a particular cell or tissue type, and are very well suited for transcript discovery in known genomic sequences. Gene prediction from genomic sequences is still far from perfect today, and the whole genome sequences of complex organisms suggest that the transcript repertoire may be quite complicated. The MPSS technology is commercialized by Lynx Therapeutics. SAGE technology is described elsewhere in this book (*UNIT 25B.6*).

Differential display (DD) technology as first described by Liang and Pardee (1992; *UNIT 25B.3*) uses one random primer and an anchored oligo-d(T) primer for amplification of cDNA fragments, which are displayed on denaturing polyacrylamide gels (i.e., sequencing gels). The major difference between DD and the AFLP cDNA technology described in this unit is that AFLP cDNA profiling allows a systematic display of cDNA fragments, with each primer combination displaying a different subset of the cDNAs (Durrant et al., 2000; Van der Biezen et al., 2000; Breyne and Zabeau, 2001; Din et al., 2001; Qin et al., 2001). This, and the smaller fragments generated by AFLP, generally yield sharper and more discrete banding patterns. Another alternative to DD is restriction enzyme analysis of differentially expressed sequences. This technology makes use of restriction enzyme cleavage sites in the cDNA and yields sharp, discrete bands like AFLP (*UNIT 25B.4*; Fischer et al., 1995; Prashar and Weismann, 1996).

The AFLP technique allows the selective amplification of subsets of genomic restriction fragments or cDNAs, which can subsequently be displayed on DNA sequencing gels. One of the characteristics of the AFLP technique is that the reaction proceeds until the primer is depleted from the reaction mixture (Vos et al., 1995). This is different from a standard PCR, where the amplification process is inhibited in the final stage of the reaction due to competition between fragment-to-fragment, reannealing, and primer-to-template annealing. This difference is probably caused by the fact that the concentration of individual AFLP fragments is much lower compared to standard PCR due to many fragments competing for the same primer set. This characteristic of the AFLP technique is of great importance for the quantitative amplification and display of transcript fragments.

Another important characteristic of the AFLP technique is the preferential amplification of *Taq*I-*Mse*I fragments compared to the *Taq*I-*Taq*I fragments and *Mse*I-*Mse*I fragments that will also result from template preparation. It is the authors' belief that the *Taq*I-*Taq*I fragments and *Mse*I-*Mse*I fragments amplify less efficiently because they contain inverted repeats at the fragment ends after adapter ligation. As a result, intramolecular self ligation of *Taq*I-*Taq*I fragments and *Mse*I-*Mse*I fragments will

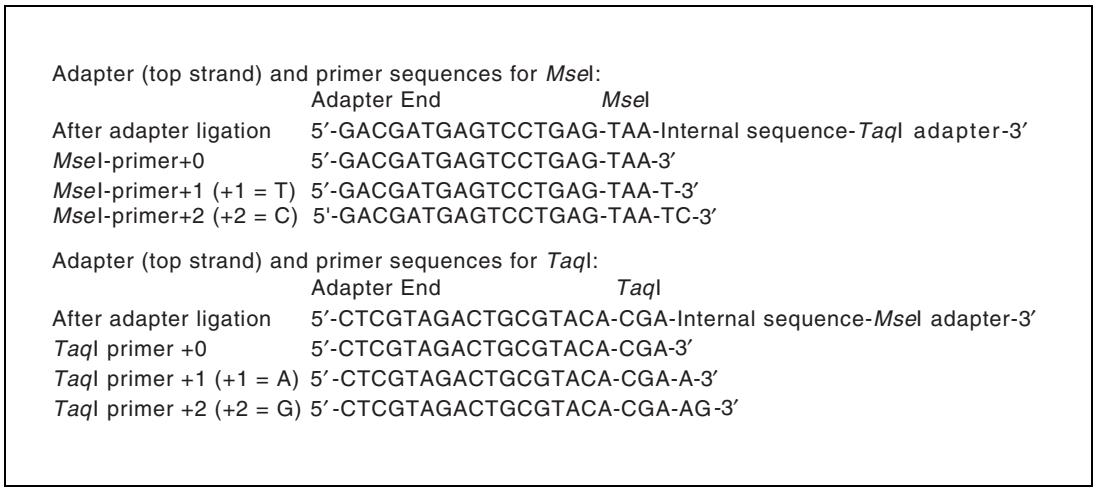
Adapter (top strand) and primer sequences for MseI:
Adapter End MseI
After adapter ligation 5′-GACGATGAGTCCTGAG-TAA-Internal sequence-TaqI adapter-3′
MseI-primer+0 5′-GACGATGAGTCCTGAG-TAA-3′
MseI-primer+1 (+1 = T) 5′-GACGATGAGTCCTGAG-TAA-T-3′
MseI-primer+2 (+2 = C) 5'-GACGATGAGTCCTGAG-TAA-TC-3′
Adapter (top strand) and primer sequences for TaqI:
Adapter End TaqI
After adapter ligation 5′-CTCGTAGACTGCGTACA-CGA-Internal sequence-MseI adapter-3′
TaqI primer +0 5′-CTCGTAGACTGCGTACA-CGA-3′
TaqI primer +1 (+1 = A) 5′-CTCGTAGACTGCGTACA-CGA-A-3′
TaqI primer +2 (+2 = G) 5′-CTCGTAGACTGCGTACA-CGA-AG-3′

Figure 25B.5.3 Schematic of primer design.

compete with primer annealing during AFLP amplification. This hypothesis is supported by the observation that amplification of *Mse*I-*Mse*I fragments is efficient when two different *Mse*I adapters are used for template preparation and two corresponding *Mse*I primers are used for amplification (P. Vos, unpub. observ.).

In the protocol outlined in this chapter, double-stranded cDNA is restriction digested with *Taq*I and *Mse*I, and adapters for these two restriction endonucleases are ligated to the resulting restriction fragments. Adapters create a target site for the AFLP primers in the subsequent amplification reactions. The adapter ligation is performed in a way that the original *Taq*I and *Mse*I sites are not restored. After adapter ligation the *Taq*I-*Mse*I restriction fragments have from 5′ to 3′ a universal sequence at the *Taq*I end (*Taq*I adapter + remnant of *Taq*I site), the original sequence between the *Taq*I and *Mse*I recognition sequence, and a second (different from *Taq*I) universal sequence at the *Mse*I end (*Mse*I adapter + remnant of *Mse*I site; Figure 25B.5.3). The primer design matches the newly created fragment ends. The use of the restriction endonuclease combination *Taq*I-*Mse*I and primers containing four selective nucleotides (two selective bases for *Taq*I and two selective bases for *Mse*I) divides the mixture of transcript fragments into 256 different fragment subsets. Each fragment subset will be amplified by a specific combination of *Taq*I and *Mse*I primers (i.e., a primer combination), and will display a small amount (i.e., ~1/256) of the transcript fragments in a specific sample. From various experiments it is known that an AFLP fragment will be detected if at least 1/1000 part of the AFLP primer is incorporated in the AFLP fragment (P. Vos unpub. observ.; P. Stanssens unpub. observ.); therefore, the detection sensitivity of the protocol described in this unit will generally be quite high. However, it should be noted that the detection sensitivity may vary from one primer combination to another as a result of the specific subset of transcript fragments that will be amplified within each primer combination.

In conclusion, the use of cDNA AFLP is an attractive technology for gene expression analysis and transcript discovery, particularly in organisms for which little or no sequence information is available. The technology is complementary to microarray based transcript imaging techniques that rely on prior characterization of the gene sequences.

Critical Parameters and Troubleshooting

AFLP analysis of genomic DNA is a very robust technology that has been used by numerous laboratories around the world for the past five years. Very few technical problems are generally encountered (Vos et al., 1995; Vos and Kuiper, 1998); however, the quality of the poly(A)$^+$ RNA and resulting ds cDNA is critical to its success. The authors advise that the protocols for poly(A)$^+$ RNA isolation from total RNA and the synthesis of ds cDNA be strictly followed.

Despite the robustness of AFLP, there are several theoretical and technical reasons why specific transcripts might not be displayed. These include (1) low transcript abundance, (2) the absence of relevant restriction enzyme sites in the transcript, and (3) features of the transcript that prevent efficient reverse transcrip-

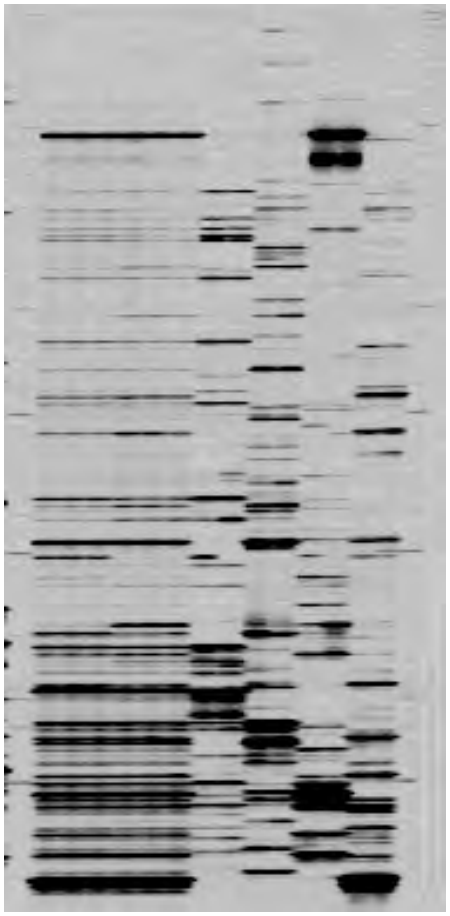

Figure 25B.5.4 cDNA fingerprint of *Aspergillus niger* that displays a very typical result for AFLP technology. Samples A1 to A3 represent three different samples which have been taken independently through the procedure of RNA isolation, cDNA synthesis, template preparation, and cDNA-AFLP reactions (notice the reproducibility). The same is true of samples B1 to B3; however, these samples were induced differently than the "A" sample sets and therefore a number of differentially expressed cDNAs are detected between the two samples. Fingerprints on the right represent +2/+2 fingerprints, and on the left corresponding +1/+2 fingerprints. The figure clearly shows that the cDNA fragments in the +2/+2 fingerprints are a subset of the cDNA fragments in the +1/+2 fingerprint.

tion (e.g., secondary structure). In the authors' experience the major cause for this is deviation from the protocol as outlined above. Annotations to the steps highlight important considerations.

The quality of the sequence gels can simply be verified by adding a "sequence ladder" to the gel. Gels that work well for sequencing will be good for AFLP profiling as well.

Anticipated Results

All experiments carried out according to the protocol outlined above will give satisfactory results. Typical transcript profiles show 50 to 100 cDNA AFLP fragments per lane (i.e., sample). The profiles should change completely when a different primer combination is used, with virtually none of the fragments being the same. Transcript profiles from the same individual will vary according to the tissue that is inspected and the conditions that are used (e.g., developmental stages, environmental factors, pathogenic infections). Figure 25B.5.4 displays an example of a typical experiment with the transcript profiles of various organisms.

Table 25B.5.1 Typical AFLP Experiment Time Course

Day 1	Poly(A)$^+$ RNA isolation and synthesis of ds cDNA
Day 2	AFLP cDNA template preparation and nonselective preamplification reactions
Day 3	Selective amplification, gel electrophoresis and overnight exposure of the gels to X-ray films or phosphoimaging screens
Day 4	Analysis of results

Time Considerations

Starting from total RNA, the following time considerations are expected for up to 96 samples:

1. Isolating poly(A)$^+$ RNA: 1 to 2 hr depending on the number of samples.
2. Synthesizing ds cDNA from the poly(A)$^+$ RNA: 5 to 6 hr.
3. Preparing the AFLP cDNA templates from the ds cDNA: 5 to 6 hr.
4. Nonselective preamplification: 3 to 4 hr (up to 96 samples).
5. Selective amplification: 3 to 4 hr (up to 96 samples).
6. Gel electrophoresis (up to 4 × 96 samples), 6 to 8 hr.

The procedure may be interrupted after each of above steps. A typical experiment time course starting from poly(A)$^+$ RNA is given in Table 25B.5.1.

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Serial Analysis of Gene Expression (SAGE): Experimental Method and Data Analysis

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ABSTRACT

Serial analysis of gene expression (SAGE) involves the generation of short fragments of DNA, or tags, from a defined point in the sequence of all cDNAs in the sample analyzed. This short tag, because of its presence in a defined point in the sequence, is typically sufficient to uniquely identify every transcript in the sample. SAGE allows one to generate a comprehensive profile of gene expression in any sample desired from as little as 100,000 cells or 1 μg of total RNA. SAGE generates absolute, rather than relative, measurements of RNA abundance levels, and this fact allows an investigator to readily and reliably compare data to those produced by other laboratories, making the SAGE data set increasingly useful as more data is generated and shared. Software tools have also been specifically adapted for SAGE tags to allow cluster analysis of both public and user-generated data. *Curr. Protoc. Mol. Biol.* 80:25B.6.1-25B.6.39. © 2007 by John Wiley & Sons, Inc.

Keywords: Genomics • mRNA • expression profiling • DNA sequencing

INTRODUCTION

This unit provides a protocol for performing serial analysis of gene expression (SAGE). SAGE involves the generation of short fragments of DNA, or tags, from a defined point in the sequence of all cDNAs in the sample analyzed. This short tag, because of its presence in a defined point in the sequence, is typically sufficient to uniquely identify every transcript in the sample. SAGE allows one to generate a comprehensive profile of gene expression in any sample desired from as few as 100,000 cells or as little as 1 μg total RNA. SAGE also allows an investigator to readily and reliably compare data to those produced by other laboratories, making the SAGE data set increasingly useful as more data are generated and shared.

Serial analysis of gene expression (SAGE), as described in the main method (see Basic Protocol 1), involves the generation of an oligonucleotide library, with each 14-bp SAGE tag representative of a discrete cDNA. Sometimes, the gene that the SAGE tag represents cannot be readily identified. Thus, a second method (see Basic Protocol 2) describes reverse cloning the 3′ end of the cognate cDNA for an unknown SAGE tag. Three additional protocols for verifying cDNA by PCR (see Support Protocol 1), optimizing ditag PCR (see Support Protocol 2), and annealing linkers (see Support Protocol 3), are also given. Finally, protocols for use of publicly available cluster analysis software designed for analysis of SAGE data are described in Basic Protocol 3.

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BASIC PROTOCOL 1

MicroSAGE

SAGE library construction involves anchoring mRNA molecules via their poly(A) tails to magnetic beads. cDNA synthesis is then conducted, and the cDNAs are cleaved with *Nla*III to completion. (MicroSAGE, which is described here, differs from conventional SAGE in that this anchoring at the 3′ end takes place prior to cDNA synthesis rather than after cDNA synthesis.) This results in the loss of all cDNA sequence 5′ to the cleavage site, and ensures that only the 3′-most *Nla*III site is exposed at the 3′ end of the cDNA. The cDNA sample is then divided into two equal pools and two sets of linkers (which contain a *Bsm*FI site, PCR primer sites, and modified 3′ bases to prevent ligation to each other) are then added by ligation. *Bsm*FI is a type IIS restriction enzyme, with a cut site 15 bp 3′ of the recognition site. The resulting cDNAs are then digested with *Bsm*FI, which results in the release of the linker, the *Nla*III site, and 10 to 11 bp 3′ of the *Nla*III site. The resulting "tags" are then blunt-ended with the Klenow fragment of DNA polymerase I, and the two separate pools of tags are ligated together via blunt-end ligation to form "ditags." These are then amplified via the PCR primer sites incorporated into the linkers and then recleaved with *Nla*III. These cleaved ditags are purified and ligated together to form concatemers of tags, which are then subcloned into plasmid vectors to create a SAGE library. Individual clones are then sequenced, and analyzed via SAGE analysis software. SAGE software identifies and discards any sets of duplicate ditags (i.e., a given combination of any two individual tags) to control for PCR amplification bias. It can also be used to prepare a tag report, listing all tags and their abundance in a given library, or a tag comparison file, listing the tag abundances in a number of different libraries.

An overview of the microSAGE protocol is shown in Figure 25B.6.1.

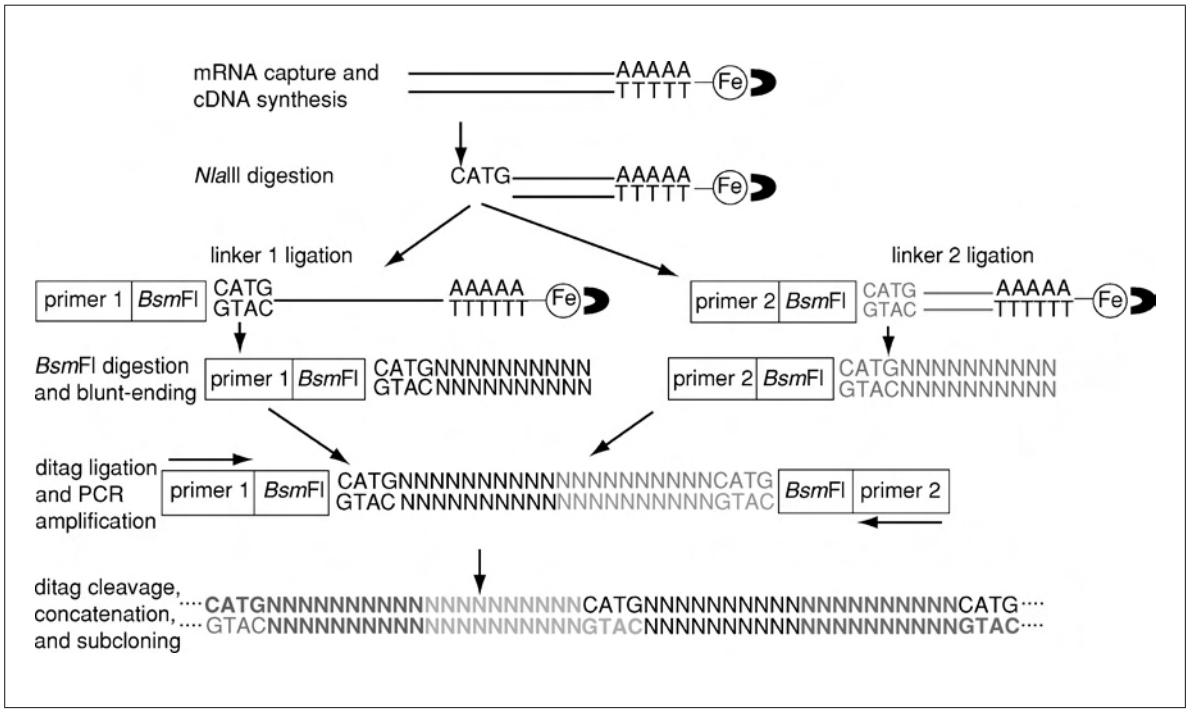


Figure 25B.6.1 The steps of a SAGE experiment.

Materials

Dynabeads mRNA DIRECT kit (Dynal Biotech):
  Dynabeads oligo$(dT)_{25}$
  Lysis/binding buffer
  Washing buffer A: add 1 μl 20 mg/ml molecular-biology-grade glycogen (Roche Diagnostics) per milliliter
  Washing buffer B
Cells or tissue of interest
SuperScript Choice System cDNA synthesis kit (Invitrogen):
  5× first-strand buffer
  DEPC-treated (*UNIT 4.1*) double-distilled water (DEPC ddH_2O)
  1× first-strand buffer: dilute from 5× stock in DEPC ddH_2O
  0.1 M DTT
  10 mM dNTP
  200 U/μl SuperScript II reverse transcriptase
  5× second-strand buffer
  10 U/μl *E. coli* DNA ligase
  10 U/μl *E. coli* DNA polymerase I
  2 U/μl *E. coli* RNase H
  1× and 5× T4 DNA ligase buffer
  1 U/μl T4 DNA ligase
0.5 M EDTA, pH 8.0 (*APPENDIX 2*)
1× BW buffer (see recipe)/2× BSA (New England Biolabs)/0.1% (w/v) SDS
1× BW buffer/2× BSA
1× NEBuffer 4 (New England Biolabs)/2× BSA
LoTE buffer (see recipe)
100× BSA (New England Biolabs)
10 U/μl *Nla*III and 10× NEBuffer 4 (New England Biolabs): store at −80°C
1× BW buffer/2× BSA/1% (v/v) Tween 20
Annealed linkers (see Support Protocol 3)
5 U/μl (high-concentration) T4 DNA ligase (Invitrogen)
2 U/μl *Bsm*FI (New England Biolabs)
PC8 (see recipe)
SeeDNA (Amersham Pharmacia Biotech)
3:1 solution of 20 mg/ml glycogen/SeeDNA (optional)
3 M sodium acetate (*APPENDIX 2*)
70% and 100% ethanol
Klenow fragment of DNA polymerase I and 10× buffer (Amersham Pharmacia Biotech) or Roche Buffer H
3 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
10× SAGE PCR amplification buffer (see recipe)
DMSO (Sigma)
PCR primers (see recipe):
  350 ng/μl primers 1 and 2
  350 ng/μl M13 forward and reverse primers
5 U/μl Platinum *Taq* DNA polymerase (Invitrogen)
20 mg/ml glycogen (Roche Diagnostics)
7.5 M ammonium acetate (Sigma)
Dry ice/methanol bath
5× loading buffer: 50 mM EDTA/50 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)/50% (v/v) glycerol
20% (w/v) polyacrylamide/TBE minigels (Novex)
20-bp DNA ladder (GenSura)
10,000× SYBR Green I (Roche Diagnostics)

1× TBE (*APPENDIX 2*)
1-kb DNA ladder
pZErO-1 plasmid (Invitrogen)
*Sph*I and NEBuffer 2 (New England Biolabs)
TE buffer, pH 8.0 (*APPENDIX 2*)
SOC medium (*UNIT 1.8*)
0.01 ng/μl pUC19 control DNA
DH10B Electromax competent cells, −70°C (Invitrogen)
LB medium (*UNIT 1.1*; optional)
LB plates with 100 μg/ml ampicillin (*UNIT 1.1*)
10-cm zeocin-containing low-salt LB plate (see recipe)
10:1 U/μl *Taq*/*Pfu* polymerase (Stratagene)
Exonuclease I (USB)
Shrimp alkaline phosphatase (USB)
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)

0.5-, 1.5-, 2.0-ml RNase-free No-stick siliconized microcentrifuge tubes (Ambion)
Magnetic rack for 1.5-ml microcentrifuge tubes (Dynal Biotech)
Tissue homogenizer (e.g., Polytron PT1200, Brinkmann Instruments)
23-G needles and 1-ml syringes
200-μl aerosol-barrier pipet tips
16° and 65°C water baths, heat blocks, or equivalent
96-well PCR plates
50-ml conical tubes
Tabletop centrifuge with swinging-bucket rotor
Gel-loading tips
UV box and SYBR green or UV filter
0.5-ml microcentrifuge tubes with ∼0.5-mm holes in the bottom: pierce from the inside out with a 21-G needle
Spin-X centrifuge-tube filters (Costar)
Long-wavelength UV source
0.1-mm disposable microelectroporation cuvettes (Bio-Rad)
Gene Pulser electroporator (Bio-Rad) or equivalent
15-ml culture tubes

Additional reagents and equipment for determining integrity of cDNA by PCR (see Support Protocol 1), optimizing ditag PCR conditions (see Support Protocol 2), agarose gel electrophoresis (*UNIT 2.5A*), ethanol precipitation (*UNIT 2.1A*), polyacrylamide gel electrophoresis (*UNIT 2.7*) and direct sequencing of PCR products (*UNIT 15.2*)

NOTE: Prepare Dynabeads, washing solutions, and 5× first-strand mix before thawing and collecting cells.

Prepare mRNA and synthesize cDNA

1. Thoroughly resuspend Dynabeads oligo $(dT)_{25}$, transfer 100 μl to a 1.5-ml RNase-free siliconized No-stick microcentrifuge tube, and place on a magnetic rack. After ∼30 sec remove supernatant.

   This volume of beads is much more than needed, but permits easy handling.

   When removing the supernatant, always place the pipet tip at the opposite side of the tube, push the pipet tip to the bottom, and pipet very slowly, so as not to disturb the beads.

2. Resuspend beads in 500 µl lysis/binding buffer by "flicking" the tube or by gently vortexing. Leave beads in buffer until ready to add them to the cell lysate (step 4).

   In this and all subsequent washing steps, add solution to the tube while keeping it on the magnetic rack in order to minimize "drying out" of the beads. Next, close the cap, remove the tube from the magnet, and resuspend the beads. Place back on the magnetic rack for ~30 sec to collect beads at the bottom before removing wash.

3. Lyse 100,000 to 1,000,000 cells (or 2 to 10 mg tissue) in 1 ml lysis/binding buffer in a 2-ml microcentrifuge tube with a tissue homogenizer for 1 min.

   Before using the homogenizer, clean it thoroughly, rinse with 100% ethanol, and pulse in 1 liter DEPC ddH_2O.

   If necessary, remove any cellular debris that remains following homogenization by microcentrifuging 1 min at maximum speed.

4. Immediately shear genomic DNA by pressing lysed cells through a 23-G needle attached to a 1-ml syringe into the tube containing prewashed Dynabeads (step 2), from which the buffer has been removed. Incubate 3 to 5 min at room temperature with constant agitation by hand.

   Alternatively, total RNA previously isolated and stored at −80°C may be used. Total RNA (1 to 10 µg in 500 µl of lysis/binding buffer) may be added and incubated 3 to 5 min, room temperature, with constant agitation by hand. It is best to run some of the RNA on a denaturing gel to check for degradation. Visualization of sharp 28S and 18S ribosomal bands should be seen.

5. Place the tube on a magnetic rack for 2 min, then remove the supernatant.

   This supernatant can be used for a genomic DNA prep if desired.

6. Wash beads by pipetting up and down several times with a 200-µl aerosol-barrier pipet tip in the following sequence:

   Twice with 1 ml washing buffer A
   Once with 1 ml washing buffer B
   Four times with 1× first-strand buffer.

   Pipetting the beads is more efficient than flicking the tubes.

7. Resuspend beads in the following first-strand synthesis mix:

   54 µl DEPC ddH_2O
   18 µl 5× first-strand buffer
   9 µl 0.1 M DTT
   4.5 µl 10 mM dNTP.

   Heat tube 2 min at 37°C, then add 3 µl of 200 U/µl SuperScript II reverse transcriptase. Incubate 1 hr at 37°C, mixing beads every 10 min by hand. Terminate reaction by placing tube on ice.

8. Add the following components of the second-strand synthesis to the first-strand reaction, on ice, in the order shown:

   227 µl ddH_2O, prechilled
   150 µl 5× second-strand buffer
   15 µl 10 mM dNTP
   3 µl 10 U/µl *E. coli* DNA ligase
   12 µl 10 U/µl *E. coli* DNA polymerase I
   3 µl 2 U/µl *E. coli* RNase H.

   Incubate 2 hr at 16°C, mixing beads every 10 min by hand.

9. After incubation, place tubes on ice and terminate reaction by adding 100 µl of 0.5 M EDTA, pH 8.0.

10. Wash beads one time with 0.5 ml of 1× BW buffer/2× BSA/0.1% (w/v) SDS.

    The BSA appears to reduce the stickiness of the beads and improves the efficiency of the washes and the quality of the library. Extra washes with SDS can cause beads to clump severely.

11. Wash beads three times, each in 500 µl of 1× BW buffer/2× BSA. Resuspend beads in 500 µl of 1× BW buffer/2× BSA and heat 20 min at 75°C.

    This heating step is crucial as it inactivates the nuclease activity of PolI.

12. Wash three times in 500 µl of 1× BW buffer/2× BSA. Wash twice with 200 µl of 1× NEBuffer 4/2× BSA, transferring to new tubes after the first wash in NEBuffer 4/BSA and saving 5 µl of the last bead suspension.

13. Using the saved 5-µl aliquot, check the integrity of the cDNA by PCR (see Support Protocol 1), using primers for genes known to be in the cDNA used for library construction.

Cleave cDNA with anchoring enzyme (NlaIII) and ligate linkers to cDNA

14. Resuspend beads in following mix:

    171 µl LoTE buffer
    4 µl 100× BSA
    20 µl 10× NEBuffer 4
    5 µl 10 U/µl *Nla*III.

    Incubate 1 hr at 37°C.

15. After incubation, place on a magnetic rack ~30 sec, then wash beads with the following solutions by pipetting up and down several times with a 200-µl aerosol-barrier pipet tip:

    Twice with 500 µl 1× BW/2× BSA/1% Tween 20
    Four times with 500 µl 1× BW/2× BSA
    Twice with 1× T4 DNA ligase buffer.

    After final resuspension in ligase buffer, transfer 100 µl of each sample into two new 1.5-ml siliconized microcentrifuge tubes.

16. Remove last wash and resuspend beads with the following:

    5 µl LoTE buffer (both tubes)
    2 µl 5× T4 DNA ligase buffer (both tubes)
    3 µl 2 ng/µl annealed linkers 1A and 1B (only in tube 1)
    3 µl 2 ng/µl annealed linkers 2A and 2B (only in tube 2).

17. Heat tubes 2 min at 50°C then let sit for 5 to 15 min at room temperature. Add 1 µl of 5 U/µl (high-concentration) T4 DNA ligase to each tube and incubate 2 hr at 16°C. Mix beads intermittently.

Release cDNA-tags using tagging enzyme BsmFI

18. After ligation, place on a magnetic rack ~30 sec, then wash each sample two times with 500 µl of 1× BW/2× BSA/0.1% SDS each, pooling tube 1 and tube 2 together after first wash in order to minimize loss in subsequent steps.

19. Wash four times with 500 µl of 1× BW/2× BSA each and twice with 200 µl of 1× NEBuffer 4/2× BSA (transfer to new tubes after first wash in NEBuffer 4/BSA).

20. Preheat the following mix 2 min at 65°C:

    170 µl LoTE buffer
    20 µl 10× NEBuffer 4
    4 µl 100× BSA
    2 µl 2 U/µl *Bsm*FI.

    Resuspend beads in the mixture and incubate 1 hr at 65°C, mixing intermittently.

21. After incubation, microcentrifuge 2 min at maximum speed, then transfer supernatant to a new 1.5-ml microcentrifuge tube. Wash beads once with 40 µl LoTE buffer, then resuspend to a final volume of 240 µl with LoTE buffer.

    IMPORTANT NOTE: *From this point on, do not use siliconized tubes.*

22. Extract with 240 µl PC8 and ethanol precipitate with SeeDNA using the following procedure:

    a. Add 4 µl SeeDNA. Alternatively, use 4 µl of a 3:1 solution of 20 mg/ml glycogen/SeeDNA mix.
    b. Add 0.1 vol of 3 M sodium acetate (24 µl) and mix briefly.
    c. Add 2 vol of 100% ethanol (480 µl) and vortex briefly.
    d. Incubate 2 min at room temperature.
    e. Microcentrifuge 5 min at maximum speed.
    f. Wash two times with 70% ethanol and microcentrifuge again after last wash. Carefully remove residual liquid with a pipet tip and resuspend pellet in 10 µl LoTE buffer.

    SeeDNA is a brightly colored carrier molecule that allows easy visualization and maximal recovery of alcohol-precipitated DNA or RNA. The glycogen/SeeDNA mixture may be used to reduce cost.

    One may pause the protocol here and store the pellet overnight at −20°C.

Perform blunt-end digestion on released tags

23. Add the following mix to tags:

    30.5 µl ddH_2O
    5 µl 10× Klenow buffer (or Roche Buffer H)
    2.5 µl 10 mM dNTPs
    1 µl 100× BSA
    1 µl Klenow fragment of DNA polymerase I.

    Incubate 30 min at 37°C then add 190 µl LoTE buffer (240 µl final volume).

24. Extract with an equal volume of PC8 (240 µl). Transfer 200 µl into a ligase "+" tube and the remaining 40 µl into a ligase "−" tube.

25. Ethanol precipitate with 2 µl SeeDNA, 0.1 vol of 3 M sodium acetate, and 2 vol of 100% ethanol. Wash two times with 70% ethanol and centrifuge again after last wash. Carefully remove residual liquid with a pipet tip and air-dry 5 to 10 min. Resuspend pellet in 2 µl LoTE buffer.

    Do not overdry because DNA will be lost.

Ligate tags to form ditags

26. Prepare 2× ligase "+" mix as follows:

    2.5 µl 3 mM Tris·Cl, pH 7.5
    3.0 µl 5× T4 DNA ligase buffer
    2.0 µl 5 U/µl (high-concentration) T4 DNA ligase.

    Prepare a 2× ligase "−" mix with 4.5 µl of 3 mM Tris·Cl, pH 7.5 and 3.0 µl of 5× T4 DNA ligase buffer. Add 2 µl of appropriate mix to +/− ligase samples and incubate in a thermal cycler overnight (8 to 12 hr) at 16°C.

    The sample may dry out in a water bath (in 4°C cold room), thus incubation in a PCR machine/thermal cycler is preferable.

27. After ligation, add 98 µl LoTE buffer, optimize PCR conditions (see Support Protocol 2), and proceed to large-scale PCR amplification.

    Samples may be stored >1 year at −20°C.

Perform large-scale PCR amplification of ditags

28. Prepare a reaction master mix for large-scale PCR (two to three 96-well PCR plates containing 50 µl reaction per well) using the following recipe for one reaction as a guide:

    5 µl 10× SAGE PCR amplification buffer
    3 µl DMSO
    4.0 to 10 µl 10 mM dNTPs
    1 µl 350 ng/µl PCR primer 1
    1 µl 350 ng/µl PCR primer 2
    Adjust volume to 49 µl with ddH_2O
    0.7 µl 5 U/µl Platinum *Taq* DNA polymerase.

    Aliquot 49 µl of reaction mix to each well, then add 1 µl template at appropriate dilution (see Support Protocol 2).

    The authors usually use a 300-reaction PCR premix that is dispensed into 96-well plates at 50-µl per well.

    The volume of dNTPs to use is determined through optimization (see Support Protocol 2).

    Platinum Taq DNA polymerase is used because it allows for a room-temperature hot start reaction (the Taq DNA polymerase is complexed with an anti-Taq antibody that denatures when heated to 94°C).

29. Carry out the amplifications in a thermal cycler with the following parameters:

1 cycle:	2 min	94°C	(denaturation)
26 to 32 cycles:	30 sec	94°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	70°C	(extension)
1 cycle:	5 min	70°C	(final product extension).

    The number of cycles to use is determined through optimization (see Support Protocol 2). The ligase "−" sample should be amplified for 35 cycles.

    If a thermal cycler with heated lid is not available, oil can be used to prevent evaporation (see UNIT 15.1).

    Do not substitute conventional hot-start PCR for use of Platinum Taq DNA polymerase. The authors have found that yields are much lower if this is done. There is no need to refrigerate the PCR mix while setting up the reactions.

Isolate ditags

30. Pool PCR reactions into a 50-ml conical tube, adjusting volume to 11.5 ml with LoTE buffer, then extract with an equal volume of PC8.

31. Precipitate with ethanol as follows:

    11.5 ml samples
    10 μl SeeDNA
    100 μl 20 mg/ml glycogen
    5.1 ml 7.5 M ammonium acetate
    38.3 ml 100% ethanol.

    Place in a dry ice/methanol bath for 15 min. Thaw 2 min at room temperature to fully melt the solution.

32. Vortex briefly and centrifuge 30 min in a tabletop centrifuge with swinging-bucket rotor at ~3000 × *g* (4000 rpm), room temperature.

33. Wash with 5 ml of 70% ethanol, vortex, and centrifuge an additional 5 min at ~3000 × *g*, room temperature.

34. Resuspend pellet in 216 μl LoTE buffer and add 54 μl of 5× loading buffer (270 μl total).

35. Using gel-loading pipet tips, load 10 μl sample into each of 27 lanes on each of three prepoured 20% polyacrylamide/TBE minigels. Include 10 μl of a 20-bp ladder on each gel as a marker.

    It is critical not to overload the gel wells, as this can lead to linker contamination and poor separation of products.

36. Electrophorese 90 min at 160 V.

    The optimal distance for electrophoresis is ~1 cm above the bottom of the gel. The idea is to obtain maximum separation of the 102- (ditags) and 80-bp bands (linker-linker dimers) without allowing product to get too close to the edge of the gel. Depending on the apparatus and batch of TBE buffer, varying the electrophoresis time might be necessary.

37. Stain 15 min in a foil-wrapped container on a platform shaker using 2 to 5 μl of 10,000× SYBR Green I in 50 ml of 1× TBE buffer. Visualize on a UV box using a SYBR green or UV filter.

    Alternatively, use long-wavelength UV. Amplified ditags should run at 102 bp while a background band (linker-linker dimers) runs at ~80 bp.

38. Cut out only amplified ditags from the gel, and place three cut-out bands in 0.5-ml microcentrifuge tubes (nine tubes total) which have an ~0.5-mm diameter hole in the bottom.

39. Place the 0.5-ml microcentrifuge tubes in 2.0-ml siliconized microcentrifuge tubes and microcentrifuge 4 min at maximum speed.

    This serves to break up the acrylamide gel into small fragments at the bottom of the 2.0-ml microcentrifuge tube.

40. Discard 0.5-ml microcentrifuge tubes. Add 250 μl LoTE buffer and 50 μl of 7.5 M ammonium acetate to each 2.0-ml microcentrifuge tube.

    At this point, the 2.0-ml microcentrifuge tubes can remain overnight at 4°C.

41. Vortex each tube, and incubate 15 min at 65°C. Add 5 μl LoTE buffer to the membrane of each of 18 Spin-X centrifuge-tube filters.

42. Transfer contents of each tube to two Spin-X centrifuge tube filters (i.e., nine tubes transferred to 18 Spin-X centrifuge tube filters). Microcentrifuge each SpinX filter for 5 min at maximum speed. Consolidate sets of two eluates (300 µl total) and transfer to 1.5-ml microcentrifuge tubes.

    Sometimes purified 102-bp bands do not recut well with NlaIII, which seems to be related to imperfect purification from the gel. If this is a problem, run 300 µl eluate through a Qiaquick gel extraction protocol (Qiagen). Bring the volume of the extract back up to 300 µl to proceed.

43. Ethanol precipitate eluates by adding the following:

    300 µl sample
    0.5 µl SeeDNA
    1.5 µl glycogen
    133 µl 7.5 M ammonium acetate
    1000 µl 100% ethanol.

    Vortex and place in a dry ice/methanol bath for 15 min. Warm 2 min at room temperature until solution has melted, then microcentrifuge 15 min at 4°C.

44. Microcentrifuge 15 min at maximum speed. Wash two times with 75% ethanol. Resuspend each DNA tube in 10 µl LoTE buffer. Pool samples into one microcentrifuge tube (90 µl total).

    The total amount of DNA at this stage should be 10 to 20 µg.

45. Digest PCR products with *Nla*III by adding the following:

    90 µl PCR products in LoTE buffer
    226 µl LoTE buffer
    40 µl 10× NEBuffer 4
    4 µl 100× BSA
    40 µl 10 U/µl *Nla*III.

    Incubate 1 hr at 37°C.

Purify the ditags

46. Extract with an equal volume of PC8. Pool aqueous phases and transfer into 1.5-ml microcentrifuge tubes. Ethanol precipitate in dry ice as follows:

    200 µl sample
    66 µl 7.5 M ammonium acetate
    3 µl SeeDNA
    825 µl 100% ethanol.

    Vortex and place in dry ice/methanol bath for 15 min.

47. Warm 2 min at room temperature until solution has melted, then microcentrifuge 15 min at 4°C.

48. Wash once with cold 75% ethanol, removing ethanol traces with a gel-loading pipet tip. Resuspend pellet in 40 µl LoTE buffer. On ice, add 10 µl of 5× loading buffer (50 µl total).

49. Load this sample into four lanes of a 20% polyacrylamide/TBE gel, load the 20-bp ladder into a separate lane, and run ~2.5 hr at 160 V. Stain as described in step 37.

    Optimal electrophoresis time may vary somewhat. Be careful not to run the gel too long.

50. Cut out the 24- to 26-bp band from four lanes under long-wavelength UV illumination, and place two cut-out bands in each of two 0.5-ml microcentrifuge tubes which have an ~0.5-mm diameter hole in the bottom.

51. Microcentrifuge as described in step 39.

52. Discard the 0.5-ml microcentrifuge tubes. Add 250 µl LoTE buffer and 50 µl of 7.5 M ammonium acetate to each of the 2.0-ml microcentrifuge tubes. Vortex the tubes, and incubate 1 hr at 37°C.

    IMPORTANT NOTE: *Do not incubate at 65°C. This will cause the 26-bp ditags to denature. Longer incubations (even overnight) can be performed, but do not appear to result in significantly higher yields.*

53. Use four Spin-X centrifuge-tube filters to isolate eluate as described in step 42. Ethanol precipitate in three tubes (200 µl each) with the following:

    200 µl sample
    66 µl 7.5 M ammonium acetate
    2 µl SeeDNA
    3 µl glycogen
    825 µl 100% ethanol.

    Incubate 10 min in a dry ice/methanol bath, then microcentrifuge 15 min at 4°C.

54. Wash two times with cold 75% ethanol each. Resuspend each DNA sample on ice in 2.5 µl cold LoTE buffer and pool (7.5 µl total).

    It is critical to keep the purified ditags on ice until the ligation buffer is added. Ditags with a high A and T content can denature at room temperature, even in LoTE buffer.

Ligate ditags to form concatemers

55. Mix the following:

    7 µl pooled purified ditags
    2 µl 5× T4 DNA ligase buffer
    1 µl 5 U/µl (high-concentration) T4 DNA ligase.

    Incubate 1 to 3 hr at 16°C.

    Do not ligate overnight, as this will result in long concatemers that are difficult to clone. The authors usually ligate for 2 hr with good results.

    The length of ligation time depends on the quantity and purity of the ditags. Typically, several hundred nanograms of ditags are isolated and produce large concatemers when the ligation reaction is performed for 1 to 3 hr at 16°C (lower quantities or less-pure ditags will require longer ligations).

56. After completing ligation, add 2.5 µl of 5× loading buffer to the ligation reaction. Heat samples 5 min at 65°C and immediately place on ice.

    The heating step melts annealed sticky ends and is critical for obtaining a good yield of clonable concatemers.

57. Separate concatemers on a 10% to 12% polyacrylamide/TBE gel (*UNIT 2.7*). Load 1-kb DNA marker in first lane, leave one empty lane, and then load the entire concatenated sample into the third well. Run samples 45 min at 200 V.

58. Stain and visualize as described in step 37. Isolate regions of interest.

    Concatemers will form a smear on the gel with a range from ~100 bp to several kilobases.

    The authors usually isolate regions 600 to 1200 bp and 1200 to 2500 bp. These size ranges clone efficiently and yield ample sequencing information.

59. Place each gel piece into 0.5-ml microcentrifuge tubes which have an ~0.5-mm-diameter hole in the bottom.

60. Microcentrifuge as described in step 39.

61. Discard the 0.5-ml microcentrifuge tubes. Add 300 µl LoTE buffer to the gel pieces in the 2.0-ml microcentrifuge tubes. Vortex each tube, and incubate 15 min at 65°C.

    If desired, this incubation can be extended to overnight, but yields are not significantly increased.

    Note that ammonium acetate is not required for high-molecular-weight molecules.

62. Add 5 µl LoTE to the membrane of each of four Spin-X microcentrifuge-tube filters. Transfer contents of each tube to two Spin-X microcentrifuge-tube filters (four total). Microcentrifuge each Spin-X tube 5 min at maximum speed.

63. Pool eluates from two Spin-X centrifuge tube filters into one 1.5-ml microcentrifuge tube and ethanol precipitate by adding the following:

    300 µl eluate
    2 µl SeeDNA
    133 µl 7.5 M ammonium acetate
    1000 µl 100% ethanol.

    Glycogen can be substituted for SeeDNA, but the authors obtained better results when only SeeDNA was used.

64. Microcentrifuge 15 min at maximum speed. Wash two times with 70% ethanol and air dry 5 min. Resuspend purified concatemer DNA in 6 µl LoTE buffer.

Ligate the concatemers into vector

65. Digest 1 µg pZErO-1 plasmid with *Sph*I in a total volume of 10 µl by adding the following:

    1 µl pZErO-1 plasmid
    7 µl ddH_2O
    1 µl 10× NEBuffer 2
    1 µl 10 U/µl *Sph*I.

    Incubate 15 to 30 min at 37°C, then heat inactivate 10 min at 65°C. Do not digest >30 min.

    Concatemers can be cloned and sequenced in a vector of choice. The authors currently clone concatemers into a SphI-cleaved pZErO-1.

66. Check for complete digestion on an agarose gel (*UNIT 2.5A*). Dilute the cut vector with 90 µl TE buffer, pH 8.0, then extract with equal volume of PC8. Ethanol precipitate (*UNIT 2.1A*), wash two times with 70% ethanol, and resuspend in 40 µl water or TE buffer (~25 ng/µl of vector).

    The authors recommend using the linearized DNA immediately, but it may be stored for up to 2 weeks at −20°C with decreased ligation efficiency. Ligation efficiency varies beyond 2-week storage. A 2-to-5 fold increase in background is observed upon prolonged storage, due to self-ligation—i.e., no insert.

67. Mix the following ligation reaction and set up a duplicate reaction without concatamer as a control:

    6 µl purified concatemer (step 64; none in control)
    1.5 µl dH_2O (7.5 µl in control)
    1 µl 25 ng/µl pZErO plasmid cut with *Sph*I
    1 µl 10× T4 DNA ligase buffer
    1.0 µl 1 U/µl T4 DNA ligase.

Incubate 2 hr at 16°C.

Consider using 3 μl concatemers and save the rest for backup.

The manufacturer of pZErO plasmid warns that there is increased background at incubations >1 hr, which may result in breakthrough by spontaneous mutations in the ccdB death gene.

68. Bring sample volume to 200 μl with LoTE buffer. Extract with an equal volume PC8, then ethanol precipitate by mixing the following:

    200 μl sample
    133 μl 7.5 M ammonium acetate
    2 μl SeeDNA
    777 μl 100% ethanol.

69. Wash four times with 70% ethanol. Microcentrifuge briefly at maximum speed, remove 70% ethanol, and air dry 5 min. Resuspend in 10 μl LoTE buffer.

    Excess salt can cause arcing during electroporation and kill the cells.

Transfect DNA by electroporation

70. Place an appropriate number of 0.1-mm microelectroporation cuvettes and 1.5-ml microcentrifuge tubes on ice.

71. Place 1 ml SOC medium in an appropriate number of 15-ml culture tubes at room temperature.

72. Add 1 μl DNA from step 69 to 1.5-ml microcentrifuge tubes on ice. To determine transformation efficiency, add 1 μl of 0.01 ng/μl pUC19 control DNA to a tube labelled "control."

    Use 1 μl of the DNA for this transfection. The remainder of the sample is stored at −20°C.

73. Remove DH10B Electromax competent cells from −70°C and thaw on wet ice. When cells are thawed, mix cells by tapping gently.

74. Add 40 μl competent cells to each chilled 1.5-ml microcentrifuge tube containing DNA. Refreeze any unused cells in a dry ice/methanol bath for 5 min before returning to −70°C.

75. Pipet 40 μl of the cell/DNA mixture into a prechilled disposable microelectroporation cuvette (step 70). Perform electroporation with the Bio-Rad Gene Pulser electroporator at 100 Ω/25 μF/1.8 kV.

76. Transfer electroporated cells into a 15-ml culture tube and immediately add 1.0 ml SOC medium at room temperature. Shake 15 min at 225 rpm, 37°C.

    The incubation time is short because, in theory, the postelectroporation incubation period is required for expression of the antibiotic resistance gene, hence increasing transformation efficiency. However, given that the doubling time of the bacteria is ∼20 min, it is possible that the transformed bacteria may double during the incubation period, potentially skewing the library's representation of tags. With 15 min incubation prior to plating, the authors found the transformation efficiency to be 1.0×10^{10} cfu/μg pUC19, respectable when compared with the 1-hr incubation recommended by the manufacturer that resulted in 1.5×10^{10} cfu/μg pUC19.

77. Spread 100 μl of a 1:100 dilution of control cells (pUC19) in SOC or LB medium on LB plates containing 100 μg/ml ampicillin.

78. Plate 1/10 transfected bacteria onto each of ten 10-cm zeocin-containing low-salt LB plates. Incubate and analyze 12 to 16 hr later.

Insert-containing clones should have hundreds to thousands of colonies while no-insert control plates should have zero to tens of colonies.

Save all ten plates for each concatemer ligation reaction since, if insert size appears appropriate, these may be used for sequencing described below.

Check insert size by PCR

79. Prepare a reaction master mix using the following recipe for one reaction as a guide:

2.5 µl 10× SAGE PCR amplification buffer
1.25 µl DMSO
1.25 µl 10 mM dNTP
0.5 µl 350 ng/µl M13 forward PCR primer
0.5 µl 350 ng/µl M13 reverse PCR primer
18.5 µl ddH_2O
0.5 µl 10:1 U/µl *Taq*/*Pfu* DNA polymerase.

Pipet 25 µl master mix to wells of 96-well PCR plates.

Any thermostable polymerase can be used (with the appropriate buffer), but the Taq/Pfu mixture works well.

80. For each reaction, use a sterile toothpick or pipet tip to gently touch colony and then dip tip with a twirl into PCR mix.

81. Carry out the amplifications in a thermal cycler with the following parameters:

1 cycle:	2 min	95°C	(denaturation)
25 cycles:	30 sec	95°C	(denaturation)
	1 min	56°C	(annealing)
	2 min	72°C	(extension)
1 cycle:	5 min	70°C	(final product extension).

For Taq DNA polymerase-based PCR amplifications, an extension time of 0.5 to 1.0 min/kb of template amplified is sufficient, but in contrast, Pfu-based PCR amplifications require a minimum extension time of 1 to 2 min/kb of amplified template to achieve similar target synthesis.

82. Analyze on a 1.5% agarose gel at ~150 V (*UNIT 2.5A*).

For large-scale screening, use multichannel pipettors with an Owl Centipede 50-well horizontal electrophoresis system. The tips of the multichannel pipettors fit into every second well of the 50-slot comb used on the Owl Centipede rigs. Consequently, to maintain a sequential loading order for each 96-well plate, the authors prepare a separate 96-well loading plate with sample loading dye.

The authors typically get 85% to 95% of clones with inserts, of which >95% are >400 bp long. Libraries of this quality can be sequenced directly without gel screening and sorting.

Purify template and sequence amplification product

83. Use 2 µl PCR product (the exact amount will depend on the sequencing protocol and should be optimized) for clean-up using the following:

0.1 µl exonuclease I
0.1 µl shrimp alkaline phosphatase
1.8 µl 50 mM Tris·Cl, pH 8.0.

Add 2 µl clean-up mix to 2 µl DNA.

The exonuclease I degrades unincorporated primers while the alkaline phosphatase degrades unincorporated free nucleotides.

84. Perform reactions in 96-well plates on a thermal cycler, incubating 15 min at 37°C, then 15 min at 80°C. Add ddH_2O to 15 µl. Sequence PCR products directly (*UNIT 15.2*).

Use as little as 2 µl of diluted product for the sequencing reaction—optimize according to protocol. The authors run reactions on an ABI 3700 96 capillary machine, though any sequencing system may be used.

85. Download SAGE analysis software from SAGEnet (see Internet Resources) and follow easy-to-use instructions.

VERIFYING cDNA PRODUCTION BY PCR ANALYSIS

SUPPORT PROTOCOL 1

The PCR primers used to test efficiency of the reverse-transcription will depend on the species and tissue type from which the library is constructed. Working in mouse, the authors typically test a ubiquitously expressed mRNA (RPS17) and a more tissue-restricted mRNA. Design primers to be 18 to 22 bp in length and have a T_m of 55° to 60°C. T_m for the two primers should not differ by more than 1° to 2°C. The PCR product should be 300 to 700 bp in length, with a 5′ end not more than 1 kb from the 3′ end of the mRNA. The following describes the authors' method; however, conditions will have to be optimized for each primer set (see *UNIT 15.1*).

Materials *(also see Basic Protocol 1)*

350 ng/µl 5′ and 3′ primers (e.g., Integrated DNA Technology)
Bead suspension (see Basic Protocol 1, step 13)

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5A*)

1. Prepare the following PCR mixture:

5 µl 10× SAGE PCR buffer
3 µl DMSO
4 µl 10 mM dNTP mix
0.5 µl 350 ng/µl 5′ primer
0.5 µl 350 ng/µl 3′ primer
31.3 µl ddH_2O
0.7 µl 5 U/µl *Taq* DNA polymerase
5 µl bead suspension.

It is possible to test smaller aliquots of bead suspension depending on the abundance of the template.

2. Perform PCR using the following program:

Initial step:	2 min	95°C	(denaturation)
30 cycles:	30 sec	95°C	(denaturation)
	1 min	53°–58°C	(annealing)
	1 min	72°C	(extension)
Final step:	5 min	70°C	(final extension).

Annealing temperature should be 2° to 3°C lower than the lowest predicted T_m for the primers.

3. Analyze 5 µl of each PCR product on a 1.5% agarose gel in TAE buffer and visualize bands by ethidium bromide staining (*UNIT 2.5A*).

SUPPORT PROTOCOL 2

OPTIMIZING DITAG PCR AMPLIFICATION

The following protocol gives a method for optimizing ditag PCR by varying template concentration, nucleotide concentration, and number of cycles. The optimal template concentration to use is the one which gives a high yield of the 102-bp band with the least concentration of template. A clear plateau in yield should be seen with high concentrations of template. The optimal concentration of nucleotide is simply that which gives the highest yield of the 102-bp band. If none of the PCR reactions give high yields of the 102-bp band, repeat the protocol, but run one tube for 30 cycles and one for 32 cycles. The authors have found that the optimal concentration of nucleotide can vary from batch to batch and supplier to supplier, so repeated optimization may be required.

See Basic Protocol 1 for materials.

1. Prepare serial dilutions of LoTE diluted ditag reaction (see Basic Protocol 1, step 27) at 1:3, 1:9, 1:27, 1:81, and 1:243 in LoTE buffer using 10 μl reaction and 20 μl LoTE buffer (30 μl total) at each step.
2. Prepare the following PCR reaction mixture:

   5 μl 10× SAGE PCR amplification buffer
   3 μl DMSO
   1 μl 350 ng/μl PCR primer 1
   1 μl 350 ng/μl PCR primer 2
   28.3 μl ddH_2O
   0.7 μl 5 U/μl Platinum *Taq* DNA polymerase.
3. Prepare six tubes each containing 1 μl of either stock (see Basic Protocol 1, step 27) or diluted ditag reaction (1:3, 1:9, 1:27, 1:81, or 1:243). In duplicate, add 4, 7, or 10 μl of 10 mM dNTP mix (i.e., prepare two tubes of each dilution and nucleotide concentration pair). Add sufficient double-distilled water to bring the total volume to 11 μl.
4. Perform PCR as described (see Basic Protocol 1, step 29), using 26 cycles for one of the duplicate tubes and 28 for the other.
5. Remove 10 μl from each reaction and run on a prepoured 20% polyacrylamide/TBE gel, using a 20-bp ladder as a marker (10 μl of 1:5 dilution of the marker stock solution; see Basic Protocol 1, steps 35 and 36). Stain gel and visualize as described (see Basic Protocol 1, step 37).

   The amplified ditags should be 102 bp in size. A background band of equal or lower intensity (due to linker-linker dimers) occurs at ~80 bp. All other background bands should be of substantially lower intensity.

   The ligase "−" samples should not contain any amplified product of the size of the ditags, even at 35 cycles.

BASIC PROTOCOL 2

REVERSE CLONING UNKNOWN SAGE TAGS (rSAGE)

SAGE is a technique that allows a generally unbiased evaluation of cellular mRNAs on a genome-wide scale, thus providing a generally more quantitative analysis than subtractive cloning or microarray approaches. Furthermore, the sequencing of 14-bp SAGE tags has a significantly higher throughput than conventional expressed sequence tag (EST) approaches; however, the cDNA that a SAGE tag represents may not be readily identifiable due to the lack of an appropriate anchored cDNA sequence or multiple potential tag to gene matches. This protocol describes an approach, reverse-SAGE (rSAGE), by which the native 3′ sequence can be cloned from cDNA utilizing a variation of the original SAGE protocol and PCR primers based upon sequences in the SAGE tag. The advantage of this protocol is that the unknown gene is cloned using 3′ cDNA fragments

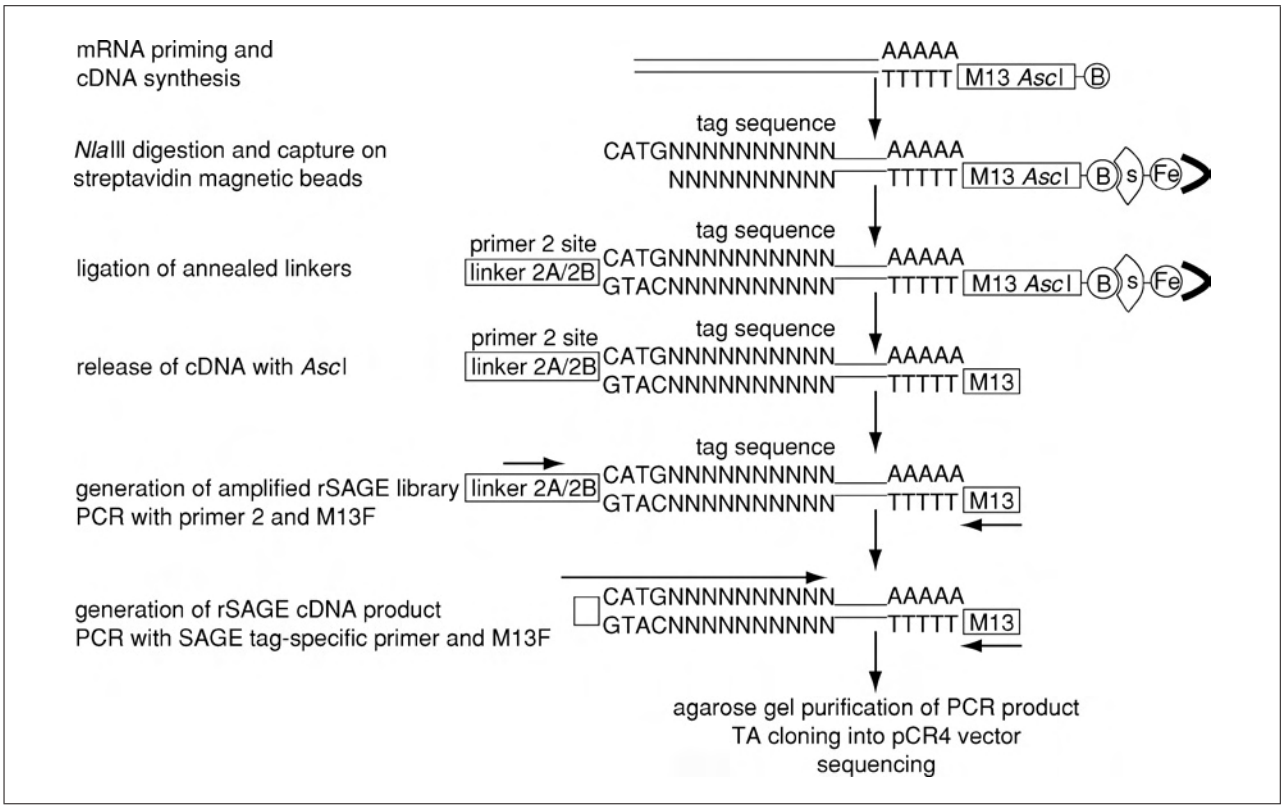


Figure 25B.6.2 Steps of an rSAGE experiment.

that are the most 3′ sequences containing the anchoring enzyme recognition sequence. This approach provides increased specificity of cloning the appropriate cognate cDNA from an anonymous SAGE tag.

Figure 25B.6.2 summarizes this procedure. The starting material is total RNA that expresses the target gene and, as a result, the anonymous SAGE tag. Double-stranded cDNA is synthesized by mRNA priming with a biotinylated poly(dT) oligonucleotide that also encodes an M13 forward priming site and an *Asc*I restriction site. The anchoring enzyme, *Nla*III, is used to cleave the cDNA and produce 3′ cDNA fragments with *Nla*III cohesive overhangs. These 3′ cDNA fragments are captured onto magnetic streptavidin Dynabeads and subsequently purified. The *Nla*III overhangs are then ligated with annealed linkers, 2A/2B, that encode a priming site for PCR primer 2, which is used for subsequent amplification. The cDNA is then released from the Dynabeads by digestion with *Asc*I restriction endonuclease. The resulting cDNA library is then amplified using PCR primer 2 and M13 forward primer (M13F). A specific rSAGE PCR product is then generated using a SAGE tag–specific primer with M13F. The SAGE tag–specific PCR product is then agarose gel purified and subsequently TA cloned into a sequencing vector.

Materials

SuperScript Choice System cDNA synthesis kit (Invitrogen):
DEPC ddH_2O
5× first-strand buffer
0.1 M DTT
10 mM dNTP
200 U/μl SuperScript II reverse transcriptase
5× second-strand buffer
10 U/μl *E. coli* DNA ligase
10 U/μl *E. coli* DNA polymerase I
2 U/μl *E. coli* RNase H

5 U/μl T4 DNA polymerase
1× and 5× T4 DNA ligase buffer
1 μg/μl gel-purified BRS1 primer (see recipe)
0.5 M EDTA, pH 7.5 (*APPENDIX 2*)
PC8 (see recipe)
SeeDNA (Amersham Pharmacia Biotech)
7.5 M ammonium acetate (Sigma)
70% and 100% ethanol
LoTE buffer (see recipe)
100× BSA (New England Biolabs)
10 U/μl *Nla*III and 10× NEBuffer 4 (New England Biolabs)
Streptavidin Dynabeads (Dynal)
1× BW buffer (see recipe)
Annealed linkers (see Support Protocol 1)
5 U/μl (high-concentration) T4 DNA ligase (Invitrogen)
1× BW buffer/1× BSA
1× NEBuffer 4/1× BSA
100× BSA
10 U/μl *Asc*I (New England Biolabs)
10× SAGE PCR buffer (see recipe)
DMSO
PCR primers (see recipe):
350 ng/μl M13 forward primer
350 ng/μl primer 2
5 U/μl Platinum *Taq* DNA polymerase (Invitrogen)
4% to 20% TBE acrylamide gel (Novex)
1-kb ladder
1× SYBR green I (Roche Diagnostics) in TBE buffer (*APPENDIX 2*)
5 M betaine: prepare monohydrate salt (Sigma) in PCR-grade ddH_2O
SAGE tag–specific primer (see recipe)
Qiaquick gel-extraction kit (Qiagen):
Qiaquick columns
EB Buffer
TOPO TA Cloning Kit with pCR2.1 vector (Invitrogen) *or* TOPO TA Cloning Kit for Sequencing with pCR4-TOPO vector (Invitrogen)

16°, 50°, and 70°C water baths, heat blocks, or equivalent
1.5-ml No-stick siliconized microcentrifuge tubes (Ambion)
Magnetic rack for 1.5-ml microcentrifuge tubes(Dynal)
1.5-ml nonsiliconized nuclease-free microcentrifuge tubes

Additional reagents and equipment for preparing total RNA (*UNIT 4.2*), agarose gel electrophoresis (*UNIT 2.5A*), and sequencing (*UNIT 7.4A*)

Synthesize cDNA

1. Prepare total RNA in DEPC ddH_2O using standard methods (e.g., *UNIT 4.2*).

   Trizol (Sigma) is the preferred method in the authors' laboratory. The same RNA with which the original SAGE library was generated would be ideal (see Basic Protocol 1, steps 3 and 4).

   It is advisable to also generate a control rSAGE library that will not express the genes of interest. As PCR cloning from the rSAGE library might generate more than one clonable band, PCR of a control rSAGE library would allow the researcher to discriminate and identify the likely rSAGE product representing the gene of interest.

2. Add 2 μl of 1 μg/μl gel-purified BRS1 primer to a nonsiliconized 1.5-ml microcentrifuge tube. Add 6 μl total RNA (5 to 10 μg total) and mix.

3. Heat mixture to 70°C for 10 min and quick chill on ice. Microcentrifuge briefly at room temperature. Prepare first-strand-synthesis mix as shown below:

   8 µl BRS1 primer/RNA
   4 µl 5× first-strand buffer
   2 µl of 0.1 M DTT
   1 µl of 10 mM dNTP.

4. Mix gently by vortexing and microcentrifuge briefly at room temperature. Incubate 2 min at 37°C, then add 5 µl of 200 U/µl SuperScript II reverse transcriptase and mix well. Incubate an additional 1 hr at 37°C.

5. After incubation, place tube on ice to terminate the reaction. Add the components of the second-strand-synthesis mixture to the first-strand reaction on ice in the order shown:

   93 µl DEPC ddH_2O, 4°C
   30 µl 5× second-strand buffer
   3 µl 10 mM dNTP
   1 µl 10 U/µl *E. coli* DNA ligase
   4 µl 10 U/µl *E. coli* DNA polymerase I
   1 µl 2 U/µl *E. coli* RNase H.

   Vortex gently to mix.

6. Incubate 2 hr at 16°C. Intermittently mix by gentle flicking. Add 2 µl 5 U/µl T4 DNA polymerase and incubate 5 min at 16°C. Place tubes on ice and terminate reaction by adding 10 µl of 0.5 M EDTA, pH 7.5.

   T4 DNA polymerase is used in the reverse-SAGE protocol to fill in 5′ overhangs generated after second-strand synthesis.

7. Add 150 µl PC8 and vortex thoroughly. Microcentrifuge 5 min at maximum speed, room temperature. Remove and save aqueous layer (~150 µl).

   Unlike microSAGE, the reverse-SAGE protocol synthesizes DNA onto unbound biotinylated oligonucleotides, making purification (i.e., phenol-chloroform extraction followed by ethanol precipitation) easier. As a result, the heat denaturation and multiple wash steps in the SAGE protocol are unnecessary.

8. Ethanol precipitate aqueous layer in a fresh standard 1.5-ml microcentrifuge tube by adding the following reagents:

   2 µl SeeDNA
   70 µl 7.5 M ammonium acetate
   500 µl 100% ethanol.

   Vortex thoroughly, then microcentrifuge 20 min at maximum speed, 4°C. Wash pellet in 70% ethanol.

9. Resuspend in 20 µl LoTE buffer.

   Samples may be stored at 4°C up to a week or frozen at −20°C for months. However, it is best to leave at 4°C overnight and resume the protocol the following day.

Cleave cDNA with anchoring enzyme (NlaIII) and ligate linkers

10. Cleave cDNA with the anchoring enzyme (*Nla*III) using the following mixture:

    20 µl cDNA (step 9)
    148 µl H_2O
    2 µl 100× BSA
    20 µl 10× NEBuffer 4
    10 µl 10 U/µl *Nla*III.

Mix and incubate 1 hr at 37°C.

It is best to proceed with prewashing the streptavidin-Dynabeads (step 11) during this incubation such that the beads will be ready for use in the subsequent steps.

11. Thoroughly resuspend Streptavidin Dynabeads, exercising care to avoid excessive vortexing as streptavidin may be sheared off the magnetic beads. Transfer 200 µl beads to a No-stick siliconized 1.5-ml microcentrifuge tube and place in a magnetic rack. After ~1 min remove supernatant. Wash beads twice in 200 µl of 1× BW then let stand in 200 µl of 1× BW until ready for use (up to several hours).

    All manipulations with Dynabeads are done using siliconized microcentrifuge tubes to avoid loss of yield due to products sticking to tube walls. All other manipulations, especially ethanol precipitations, should be done in standard microcentrifuge tubes.

    Dynabead washes are executed in the same fashion as done in the primary method (see Basic Protocol 1, step 2). Briefly, the beads are placed in the magnet 1 to 2 min. While the siliconized tube is still in the magnet, the buffer is gently pipetted off. The tube is then taken off the magnet and fresh buffer/wash is added to the tube and the beads are resuspended by agitation by hand or gentle vortexing. It is critical that the Dynabeads are not allowed to dry between the wash steps.

12. Ethanol precipitate cDNA from step 10 as described in step 8. Resuspend cDNA pellet in 200 µl of 1× BW.

13. Remove 1× BW from Dynabeads (step 11) and replace with 200 µl cDNA in BW. Mix gently by pipetting the mixture up and down. Incubate 15 min at room temperature with intermittent agitation by hand. Wash three times with 200 µl of 1× BW. Add 200 µl of 1× T4 DNA ligase buffer.

14. Prepare the following mix:

    2 µl 200 ng/µl linkers 2A and 2B (annealed)
    28 µl LoTE
    8 µl 5× T4 DNA ligase buffer.

    Remove 1× ligase buffer from the Dynabeads by pipetting and add the above mixture.

15. Mix bead slurry bound with cDNA gently, but well. Heat the tube 2 min at 50°C then incubate 15 min at room temperature.

16. Add 2 µl of 5 U/µl (high-concentration) T4 DNA ligase and incubate 2 hr at 16°C. Mix beads intermittently during ligation.

    It is best to use annealed linkers 2A/2B that are <1 month old.

Release cDNA with AscI

17. After ligation, wash beads four times with 1× BW/1× BSA. Wash in 1× NEBuffer 4/1× BSA and proceed immediately to the next step.

18. Resuspend the beads by adding the following components:

    85 µl LoTE buffer
    10 µl 10× NEBuffer 4
    2 µl 100× BSA
    2 µl 10 U/µl *Asc*I.

    Mix contents gently, but well using a pipet.

19. Incubate 1 hr at 37°C, agitating intermittently by hand every 15 min.

20. After digestion, collect supernatant carefully with a magnet. Place supernatant into a fresh nonsiliconized microcentrifuge tube. Add 50 µl LoTE to sample. Extract with PC8 as described in step 7.

21. High-concentration ethanol precipitate by combining the following:

    150 µl sample
    2 µl SeeDNA
    70 µl 7.5 M ammonium acetate
    500 µl 100% ethanol.

    Microcentrifuge 20 min at full speed, room temperature. Wash with 70% ethanol and resuspend in 25 µl LoTE.

    This is the concentrated rSAGE product, which may be stored indefinitely at −20°C. Avoid repeated freeze-thaw.

Amplify rSAGE-library dilutions by PCR

22. Make several dilutions of rSAGE product in LoTE.

    Usually 1 µl of 1:25, 1:50, and 1:100 dilutions are recommended for PCR. Due to frequent variations in yield, this can vary widely. These dilutions are good starting point, however.

23. Prepare the following PCR reaction:

    1 µl rSAGE dilution
    5 µl 10× SAGE PCR buffer
    3 µl DMSO
    3 µl 10 mM dNTPs
    1 µl 350 µg/µl M13 forward primer
    1 µl 350 µg/µl primer 2
    36 µl ddH_2O
    1 µl 5 U/µl Platinum *Taq* DNA polymerase.

    Repeat for all dilutions.

24. Use the following PCR cycling conditions:

Initial step:	2 min	94°C	(denaturation)
25 cycles:	45 sec	94°C	(denaturation)
	1 min	57°C	(annealing)
	1 min	70°C	(extension)
1 cycle:	5 min	70°C	(fill-in)
Final step:	indefinite	4°C	(hold).

25. Analyze 10 µl of each PCR product on a 4% to 20% Novex TBE acrylamide gel along with 1-kb ladder. Stain with 1× SYBR Green I in TBE buffer for 30 min and visualize under UV light.

    A smear predominantly in the 200 to 500 bp range should be observed. Choose the highest rSAGE dilution that gives reliable results. The authors usually use the amplified 1:50 dilution of the rSAGE product. Amplified rSAGE libraries may be stored at −20°C for months.

PCR amplify using SAGE tag–specific primer and M13F primer

26. Prepare the following PCR mixture per reaction:

    1 µl amplified rSAGE library (step 24)
    5 µl 10× SAGE PCR buffer
    2.5 µl DMSO
    3 µl 10 mM dNTPs
    10 µl 5 M betaine
    1 µl 350 µg/µl M13 forward primer
    1 µl 350 µg/µl SAGE tag–specific primer
    25.5 µl H_2O
    1 µl 5 U/µl Platinum *Taq*.

    See Critical Parameters and Troubleshooting for a discussion of SAGE tag–specific primers.

27. Amplify under the following PCR cycling conditions:

Initial step:	2 min	93°C	(denaturation)
1 cycle:	30 sec	93°C	(begin touchdown)
	1 min	60°C	
	1 min	70°C	
15 cycles:	30 sec	93°C	(touchdown cycles)
	1 min	60° − 1°C/cycle	
	1 min	70°C	
30 cycles:	30 sec	93°C	(amplification cycles)
	1 min	44°C	
	1 min	70°C	
1 cycle:	5 min	70°C	(fill-in)
Final step:	indefinite	4°C	(hold).

    These PCR cycling conditions are only guidelines that happen to work well for most SAGE tag–specific primers. A prolonged touchdown is pivotal for the specificity of priming. Optimal annealing temperatures may vary depending upon the nucleotide makeup of the SAGE tag–specific primer. Therefore, the touchdown annealing temperature should begin at least 10°C above the predicted oligonucleotide melting point (T_m). Over the 15 touchdown cycles, the annealing temperature should, by −1°C increments, settle upon the predicted SAGE-tag-specific primer's annealing temperature, where the rest of the 30 amplification cycles will proceed. It is not advisable to go below an annealing temperature of 40°C, regardless of how low the oligonucleotide T_m might be. Despite the apparent numerous amplification cycles used in this prolonged touchdown approach, the Taq polymerase remains very much active, mostly attributable to the protective effects of high-concentration betaine. See Critical Parameters and Troubleshooting for further discussion.

28. Visualize 5 µl of the PCR products on a 1.5% TBE agarose gel (*UNIT 2.5A*).

    The expected amplicons are usually between 100 to 400 bp, sometimes larger or smaller. Sometimes multiple bands may be amplified. If a control rSAGE amplified library was constructed, the band that is more intense in the experimental rSAGE library should be selected for further characterization. Often, multiple closely sized bands are amplification products of the same cDNA, attributable to variable oligo-dT priming along the poly(A) tract during reverse transcription.

29. Load 25 µl of PCR products into a 1.5% TBE agarose gel and electrophorese until individual bands can be resolved. Carefully excise the amplicon in the smallest agarose piece possible without sacrificing yield and place into a preweighed microcentrifuge tube.

30. Purify PCR product using the Qiaquick gel-extraction kit according to manufacturer's instructions. Elute Qiaquick columns with 30 µl EB Buffer. Proceed immediately to cloning using 4 µl eluant and the TOPO TA Cloning Kit or Cloning Kit for Sequencing per manufacturer's instructions.

    If the only goal for the rSAGE procedure is to sequence the cDNA fragment, then the standard TA cloning vector pCR2.1 (Invitrogen) should suffice. However, if there are future plans for in vitro transcription of the cloned cDNAs, then it is advisable to use the TA cloning vector pCR4-TOPO (Invitrogen), which has both T7 and T3 RNA polymerase recognition sequences flanking the multiple cloning site.

    IMPORTANT NOTE: *After TOPO TA cloning, do not use the M13 forward primer for subsequent colony PCR or cycle sequencing, as the M13 forward site will be embedded in the cloned cDNA. The M13 forward primer will not discriminate between M13 forward sites in the cDNA clone and the vector.*

31. Sequence TA cloning products using conventional methods (e.g., *UNIT 7.4A*).

PHOSPHORYLATING AND ANNEALING LINKERS

SUPPORT PROTOCOL 3

It is critical that the linkers be both annealed into double-stranded products and efficiently phosphorylated prior to ligation onto *Nla*III-digested cDNAs during SAGE-library construction. Even if linkers are ordered prephosphorylated, it is critical to test the efficiency of linker phosphorylation by self-ligation prior to SAGE library construction so as not to lose precious time and material. The following protocol details linker phosphorylation, annealing, and self ligation.

Additional Materials *(also see Basic Protocol 1)*

Linkers 1A, 1B, 2A, and 2B (see recipe)
10× kinase buffer (New England Biolabs)
10 mM ATP
10 U/µl T4 polynucleotide kinase (New England Biolabs)

Phosphorylate linkers

1. If linkers 1B and 2B are not already phosphorylated on their 5′ ends, prepare the following mixture:

    9 µl 350 ng/µl linker 1B or 2B
    6 µl LoTE buffer
    2 µl 10× kinase buffer
    2 µl 10 mM ATP
    1 µl 10 U/µl T4 polynucleotide kinase.

    Incubate 30 min at 37°C, then heat inactivate 15 min at 65°C.

Anneal linkers

2. Add 9 µl of 350 ng/µl linker 1A to 20 µl phosphorylated linker 1B.
3. Add 9 µl of 350 ng/µl linker 2A to 20 µl phosphorylated linker 2B.
4. Perform the following incubations on each linker pair:

    2 min at 95°C
    10 min at 65°C
    10 min at 37°C
    20 min at room temperature.

5. Dilute to 2 ng/µl with LoTE prior to use in SAGE-library construction.

Perform and check ligation

6. Prepare the following ligation reaction:

   0.5 µl annealed undiluted 350 ng/ml linker 1A + phosphorylated linker 1B (step 4)
   0.5 µl annealed undiluted 350 ng/ml linker 2A + phosphorylated linker 2B (step 4)
   7 µl H_2O
   1 µl 10× T4 DNA ligase buffer
   1 µl 5 U/µl (high-concentration) T4 DNA ligase buffer.

   Incubate 4 hr at 16°C.

   All linkers, whether ordered prephosphorylated or phosphorylated in-house, should be checked for self-ligation.

7. Analyze product on a prepoured 20% polyacrylamide/TBE gel. Visualize as described (see Basic Protocol 1, step 37).

   Phosphorylated linkers should allow linker-linker dimers (80 to 100 bp) to form after ligation, while nonphosphorylated linkers will prevent self-ligation. Only linker pairs that self-ligate >70% should be used in further steps.

BASIC PROTOCOL 3

USING THE SAGE DATA ANALYSIS APPLICATION

The SAGE Data Analysis Application is a statistical computational program implementing a Poisson-based algorithm for analysis of SAGE data (Cai et al., 2004). The application allows users to compare two or multiple SAGE libraries, and to perform cluster analysis. The purpose of cluster analysis is to group tags (i.e., genes) with significant changes in expression levels that behave similarly under different conditions. It has been applied in a number of genomics studies in mouse retinal development (Blackshaw et al., 2004), fetal gut development (Lepourcelet et al., 2005), and diseases, such as cancer (Allinen et al., 2004; Lepourcelet et al., 2005).

There are two user platforms for the SAGE Data Analysis Application: one is an online Web-based application and the other is a Microsoft Windows desktop-based application (stand-alone version; can be downloaded from *http://genome.dfci.harvard.edu/sager/*). Both platforms perform the same set of analyses, the difference being that the Web-based application does not require users to download and install the application onto a local computer. All data analyses are performed interactively. The potential drawback of the Web-based application is that users need to submit their SAGE data onto the online application Web server, which may risk the exposure of data to the public. If data security is a concern, the authors recommend that users use the Windows desktop-based application. The instructions in this protocol describe use of the online version.

Materials

Hardware

Computer with Internet access

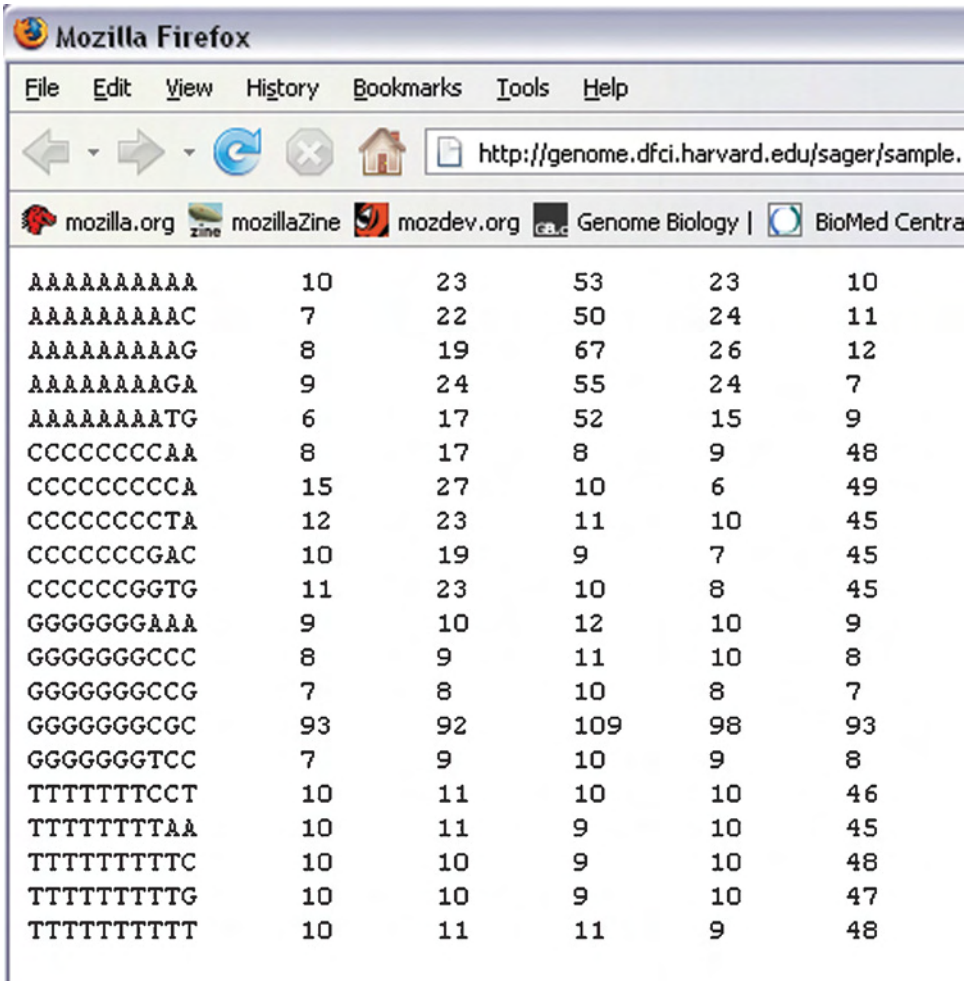
Software

An up-to-date Internet browser, such as Internet Explorer (*http://www.microsoft.com/ie*); Netscape (*http://browser.netscape.com*); Firefox (*http://www.mozilla.org/firefox*); or Safari (*http://www.apple.com/safari*).

Files

Raw SAGE data should be in tab-delimited text format with tags in rows and SAGE libraries in columns. For security purposes, the header line identifying the data in each column has been removed (see Fig. 25B.6.3). The SAGE Data Analysis Application requires that the data file be sorted by the tag sequence column (see the first column in Fig. 25B.6.3). In a Unix system this can be done with the "sort" command, and in Microsoft Windows system this can be done by choosing from the menu "Records" −> "Sort" in Microsoft Access or "Data" −> "Sort" in Excel. After sorting, export or save data as a tab-delimited text file. If the Cluster Analysis module is used, the columns that contain tag counts for all libraries in the data file must be next to each other, i.e., if the libraries start from the second column and there are 5 libraries, the 2nd through 6th columns should be the columns for tag counts from each individual SAGE library (see Fig. 25B.6.3).

NOTE: The data file can have as many extra columns as desired. As long as the correct column numbers are specified for the tag counts and first library the program should work.


Mozilla Firefox
File Edit View History Bookmarks Tools Help
http://genome.dfci.harvard.edu/sager/sample.
mozilla.org mozillaZine mozdev.org Genome Biology | BioMed Centra

AAAAAAAAAA	10	23	53	23	10
AAAAAAAAAC	7	22	50	24	11
AAAAAAAAAG	8	19	67	26	12
AAAAAAAAGA	9	24	55	24	7
AAAAAAAATG	6	17	52	15	9
CCCCCCCCAA	8	17	8	9	48
CCCCCCCCCA	15	27	10	6	49
CCCCCCCCTA	12	23	11	10	45
CCCCCCCGAC	10	19	9	7	45
CCCCCCGGTG	11	23	10	8	45
GGGGGGGAAA	9	10	12	10	9
GGGGGGGCCC	8	9	11	10	8
GGGGGGGCCG	7	8	10	8	7
GGGGGGGCGC	93	92	109	98	93
GGGGGGGTCC	7	9	10	9	8
TTTTTTTCCT	10	11	10	10	46
TTTTTTTTAA	10	11	9	10	45
TTTTTTTTTC	10	10	9	10	48
TTTTTTTTTG	10	10	9	10	47
TTTTTTTTTT	10	11	11	9	48

Figure 25B.6.3 Screen shot of a sample SAGE data file. SAGE data file needs to be in tab-delimited format. All columns of SAGE libraries (tag counts) need to be arranged next to each other. Column 1 is SAGE tag, columns 2 to 6 are tag counts for five different SAGE libraries. For online version, the column headers are removed to keep data unidentifiable by other users.

Uploading a data file

1. Navigate to the home page for the SAGE Data Analysis Application (shown in Fig. 25B.6.4) at *http://genome.dfci.harvard.edu/sager/*. Upload a tab-delimited data file by clicking "Browse" under "Step 1." Navigate to the data file, select it and then click "Send" (Fig. 25B.6.4).

   A new screen appears showing your data alongside some other previously uploaded data sets.

2. Select the data file of interest under "Step 2" on the screen.

   The choice of data file is confirmed and two calculation options are given (Fig. 25B.6.5) for significance analysis (Step 3a) and for cluster analysis (Step 3b).

Performing significance analysis

Significance analysis allows users to compare two or more different libraries and calculate *P* values. The description of the algorithm used for Poisson-based significance analysis is in the Appendix at the end of this unit.

SAGE Data Analysis Using A Poisson Approach
Li Cai[1], Haiyan Huang[2], Seth Blackshaw[3], Jun S. Liu[4], Constance L. Cepko[3], and Wing H. Wong[2,4*]
(The first two authors contributed equally to this work)
1. Department of Research Computing, Dana-Farber Cancer Institute
2. Department of Biostatistics, Harvard School of Public Health
3. Department of Genetics, Harvard Medical School
4. Department of Statistics, Harvard University
Step 1:
Upload a Tab-Delimited Data File:
Click Here to View A Sample SAGE Data File
Browse...
Send
Step 2:
Select a Data File for SAGE Data Analysis:
Significance Analysis
Clustering Analysis
SAGEmap
released: Oct 25 -- Human; Oct 25 -- Mouse.
Optional: click "SAGEmap" icon to update SAGEmaps (Hs & Mm) from NCBI.
Download SAGE Data Analysis Application
Windows Version 2.0 (30Mb); Old version: V1.0 (31Mb)
Linux, 29Mb; please read installation instructions.
Just in case you deleted the following files by accident, here they are:
Download Tutorial Material
Download "Accepted_File_Types.txt"
Download "marker.txt"
Bug Reports, Comments or Suggestions
© Li Cai, et al. 2003 -

Figure 25B.6.4 Screen shot of the main page of the online version of the SAGE Data Analysis Application.

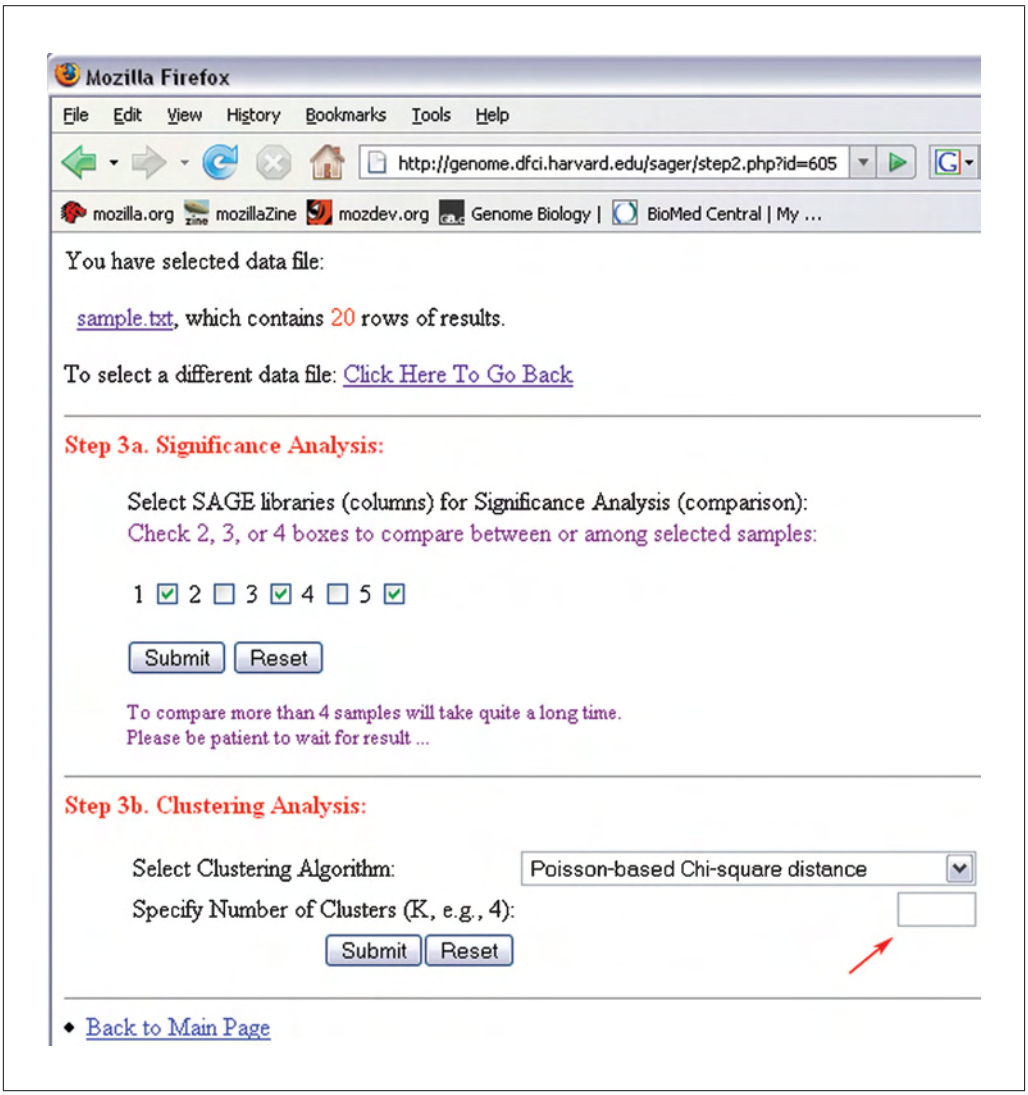
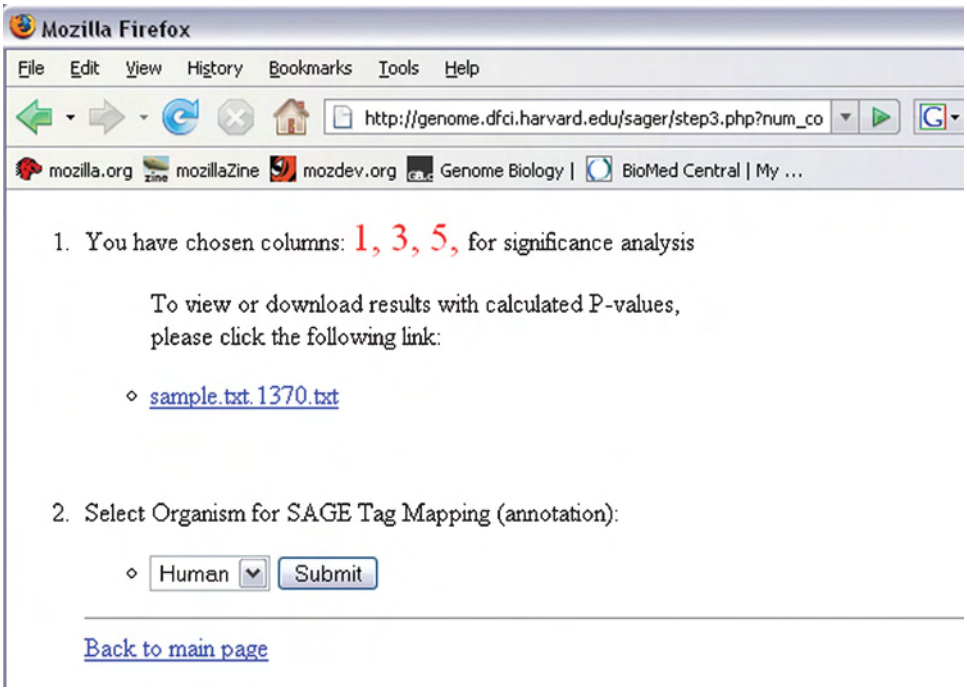

Mozilla Firefox
File Edit View History Bookmarks Tools Help
http://genome.dfci.harvard.edu/sager/step2.php?id=605
mozilla.org mozillaZine mozdev.org Genome Biology | BioMed Central | My ...
You have selected data file:
sample.txt, which contains 20 rows of results.
To select a different data file: Click Here To Go Back
Step 3a. Significance Analysis:
Select SAGE libraries (columns) for Significance Analysis (comparison):
Check 2, 3, or 4 boxes to compare between or among selected samples:
1 2 3 4 5
Submit Reset
To compare more than 4 samples will take quite a long time.
Please be patient to wait for result ...
Step 3b. Clustering Analysis:
Select Clustering Algorithm:
Poisson-based Chi-square distance
Specify Number of Clusters (K, e.g., 4):
Submit Reset
Back to Main Page

Figure 25B.6.5 Screen shot for SAGE data significance analysis.

3. Click the numbered boxes to select SAGE libraries under "Step 3a." As shown in Figure 25B.6.5, boxes "1", "3", and "5" are selected for significance analysis.
4. Click "Submit." A new screen appears (Fig. 25B.6.6).
5. Click the link to the result file ("sample.txt.1370.txt" in this example) to view or download results with calculated *P* values. The result is shown in Figure 25B.6.7. The last column contains the calculated *P* values. The result file is a tab-delimited text file. Users can open the result file in Microsoft Excel or Access. Result data can be sorted by *P* value to allow the selection of tags that are most significantly differentially expressed. The smaller the *P* value, the more significantly differentially expressed the tag.
6. To annotate the data, select an organism for SAGE tag gene mapping (Fig. 25B.6.6). Click "Submit." The annotated results appears on screen (Fig. 25B.6.8).

Performing cluster analysis

Cluster analysis is more appropriate for multiple SAGE library data sets rather than simple pair-wise comparisons between libraries. Cluster analysis allows users to select several different algorithms (distances), including Poisson-based (PoissonC), Pearson correlation (PearsonC), and Euclidean, etc., to group SAGE data into a user-defined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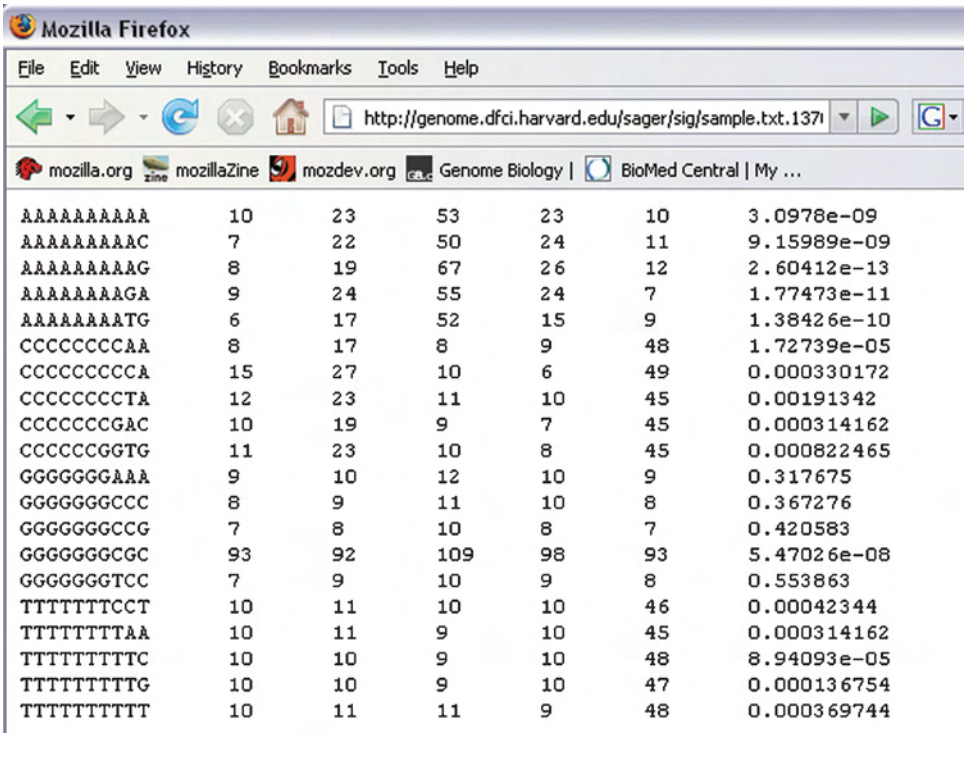
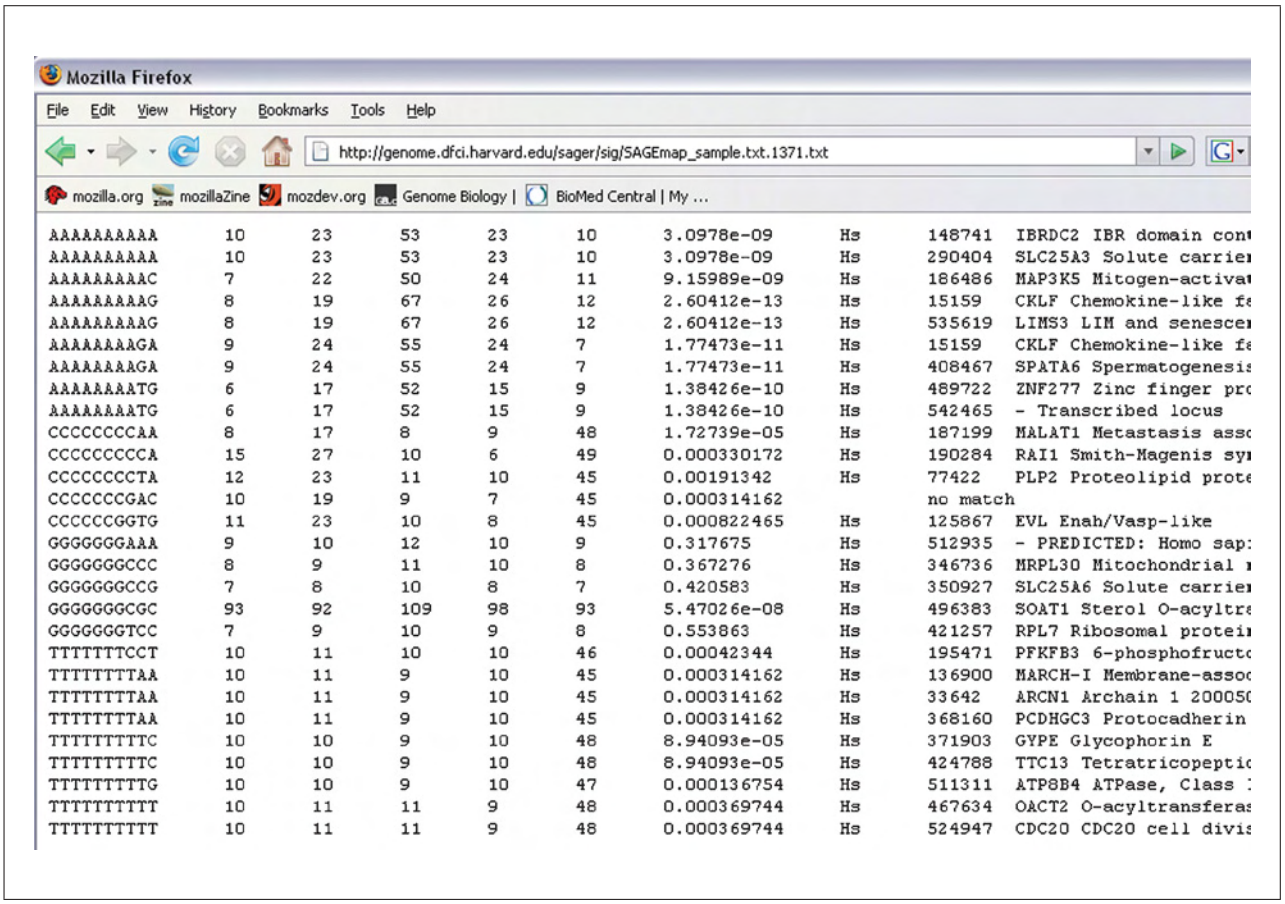
File Edit View History Bookmarks Tools Help
http://genome.dfci.harvard.edu/sager/step3.php?num_co
mozilla.org mozillaZine mozdev.org Genome Biology | BioMed Central | My ...
1. You have chosen columns: 1, 3, 5, for significance analysis
To view or download results with calculated P-values, please click the following link:
sample.txt.1370.txt
2. Select Organism for SAGE Tag Mapping (annotation):
Human
Submit
Back to main page

Figure 25B.6.6 Selection of libraries 1, 3, and 5 for significance analysis.


Mozilla Firefox
File Edit View History Bookmarks Tools Help
http://genome.dfci.harvard.edu/sager/sig/sample.txt.137
mozilla.org mozillaZine mozdev.org Genome Biology | BioMed Central | My ...

AAAAAAAAAA	10	23	53	23	10	3.0978e-09
AAAAAAAAAC	7	22	50	24	11	9.15989e-09
AAAAAAAAAG	8	19	67	26	12	2.60412e-13
AAAAAAAAGA	9	24	55	24	7	1.77473e-11
AAAAAAAATG	6	17	52	15	9	1.38426e-10
CCCCCCCCAA	8	17	8	9	48	1.72739e-05
CCCCCCCCCA	15	27	10	6	49	0.000330172
CCCCCCCCTA	12	23	11	10	45	0.00191342
CCCCCCCGAC	10	19	9	7	45	0.000314162
CCCCCCGGTG	11	23	10	8	45	0.000822465
GGGGGGGAAA	9	10	12	10	9	0.317675
GGGGGGGCCC	8	9	11	10	8	0.367276
GGGGGGGCCG	7	8	10	8	7	0.420583
GGGGGGGCGC	93	92	109	98	93	5.47026e-08
GGGGGGGTCC	7	9	10	9	8	0.553863
TTTTTTTCCT	10	11	10	10	46	0.00042344
TTTTTTTTAA	10	11	9	10	45	0.000314162
TTTTTTTTTC	10	10	9	10	48	8.94093e-05
TTTTTTTTTG	10	10	9	10	47	0.000136754
TTTTTTTTTT	10	11	11	9	48	0.000369744

Figure 25B.6.7 Results from the significance analysis. Column 1 is the SAGE tag, columns 2 to 6 are five different SAGE libraries, column 7 is calculated *P* value.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http://genome.dfci.harvard.edu/sager/sig/SAGEmap_sample.txt.1371.txt
mozilla.org mozillaZine mozdev.org Genome Biology | BioMed Central | My ...
AAAAAAAAAA 10 23 53 23 10 3.0978e-09 Hs 148741 IBRDC2 IBR domain cont
AAAAAAAAAA 10 23 53 23 10 3.0978e-09 Hs 290404 SLC25A3 Solute carrier
AAAAAAAAAC 7 22 50 24 11 9.15989e-09 Hs 186486 MAP3K5 Mitogen-activat
AAAAAAAAAG 8 19 67 26 12 2.60412e-13 Hs 15159 CKLF Chemokine-like fa
AAAAAAAAAG 8 19 67 26 12 2.60412e-13 Hs 535619 LIMS3 LIM and senescer
AAAAAAAAGA 9 24 55 24 7 1.77473e-11 Hs 15159 CKLF Chemokine-like fa
AAAAAAAAGA 9 24 55 24 7 1.77473e-11 Hs 408467 SPATA6 Spermatogenesis
AAAAAAAATG 6 17 52 15 9 1.38426e-10 Hs 489722 ZNF277 Zinc finger pro
AAAAAAAATG 6 17 52 15 9 1.38426e-10 Hs 542465 - Transcribed locus
CCCCCCCCAA 8 17 8 9 48 1.72739e-05 Hs 187199 MALAT1 Metastasis asso
CCCCCCCCCA 15 27 10 6 49 0.000330172 Hs 190284 RAI1 Smith-Magenis syn
CCCCCCCCTA 12 23 11 10 45 0.00191342 Hs 77422 PLP2 Proteolipid prote
CCCCCCCGAC 10 19 9 7 45 0.000314162 no match
CCCCCCGGTG 11 23 10 8 45 0.000822465 Hs 125867 EVL Enah/Vasp-like
GGGGGGGAAA 9 10 12 10 9 0.317675 Hs 512935 - PREDICTED: Homo sapi
GGGGGGGCCC 8 9 11 10 8 0.367276 Hs 346736 MRPL30 Mitochondrial r
GGGGGGGCCG 7 8 10 8 7 0.420583 Hs 350927 SLC25A6 Solute carrier
GGGGGGGCGC 93 92 109 98 93 5.47026e-08 Hs 496383 SOAT1 Sterol O-acyltra
GGGGGGGTCC 7 9 10 9 8 0.553863 Hs 421257 RPL7 Ribosomal protein
TTTTTTTCCT 10 11 10 10 46 0.00042344 Hs 195471 PFKFB3 6-phosphofructo
TTTTTTTTAA 10 11 9 10 45 0.000314162 Hs 136900 MARCH-I Membrane-assoc
TTTTTTTTAA 10 11 9 10 45 0.000314162 Hs 33642 ARCN1 Archain 1 200050
TTTTTTTTAA 10 11 9 10 45 0.000314162 Hs 368160 PCDHGC3 Protocadherin
TTTTTTTTTC 10 10 9 10 48 8.94093e-05 Hs 371903 GYPE Glycophorin E
TTTTTTTTTC 10 10 9 10 48 8.94093e-05 Hs 424788 TTC13 Tetratricopeptic
TTTTTTTTTG 10 10 9 10 47 0.000136754 Hs 511311 ATP8B4 ATPase, Class
TTTTTTTTTT 10 11 11 9 48 0.000369744 Hs 467634 OACT2 O-acyltransferas
TTTTTTTTTT 10 11 11 9 48 0.000369744 Hs 524947 CDC20 CDC20 cell divis

Figure 25B.6.8 Annotated results after tag matching with SAGEmap. Column 1 is the SAGE tag, columns 2 to 6 are five different SAGE libraries, column 7 is calculated *P* value, column 8 is organism (Hs = *homo sapiens*), column 9 is unigene ID, column 10 is gene symbol and gene description.

number of clusters (k). We have found that Poisson-based clustering is generally most robust algorithm for analyzing SAGE data (Cai et al., 2004). The number of clusters cannot be more than the number of tags (genes) contained in the data file. It is recommended that users test a range of values for k. A more detailed discussion of how to set the value for k is found in (Hartigan, 1975).

To start cluster analysis, users begin with "Step 3b" as shown in Figure 25B.6.5.

7. Select the clustering algorithm from the pull-down menu, as indicated by the arrow in Figure 25B.6.5.
8. Enter the desired value in the "Specify Number of Clusters" box.
9. Click "Submit."

   The run time is usually <1 min, but will vary depending on how large the dataset is. For example, for a large dataset containing >4000 unique tags, the run time could be as long as half of an hour. When clustering is finished a new screen appears, similar to that shown in Figure 25B.6.6.

10. Select an organism for annotation, then click "Submit." A screen with graphs appears. To view members of a cluster, click on the individual graphs. A new window appears with all members in the clicked cluster (Fig. 25B.6.9).
11. To save graphs, right click on individual graphs. Select "Save Image As . . ." from the menu. The user then selects a directory where the graph is to be saved.

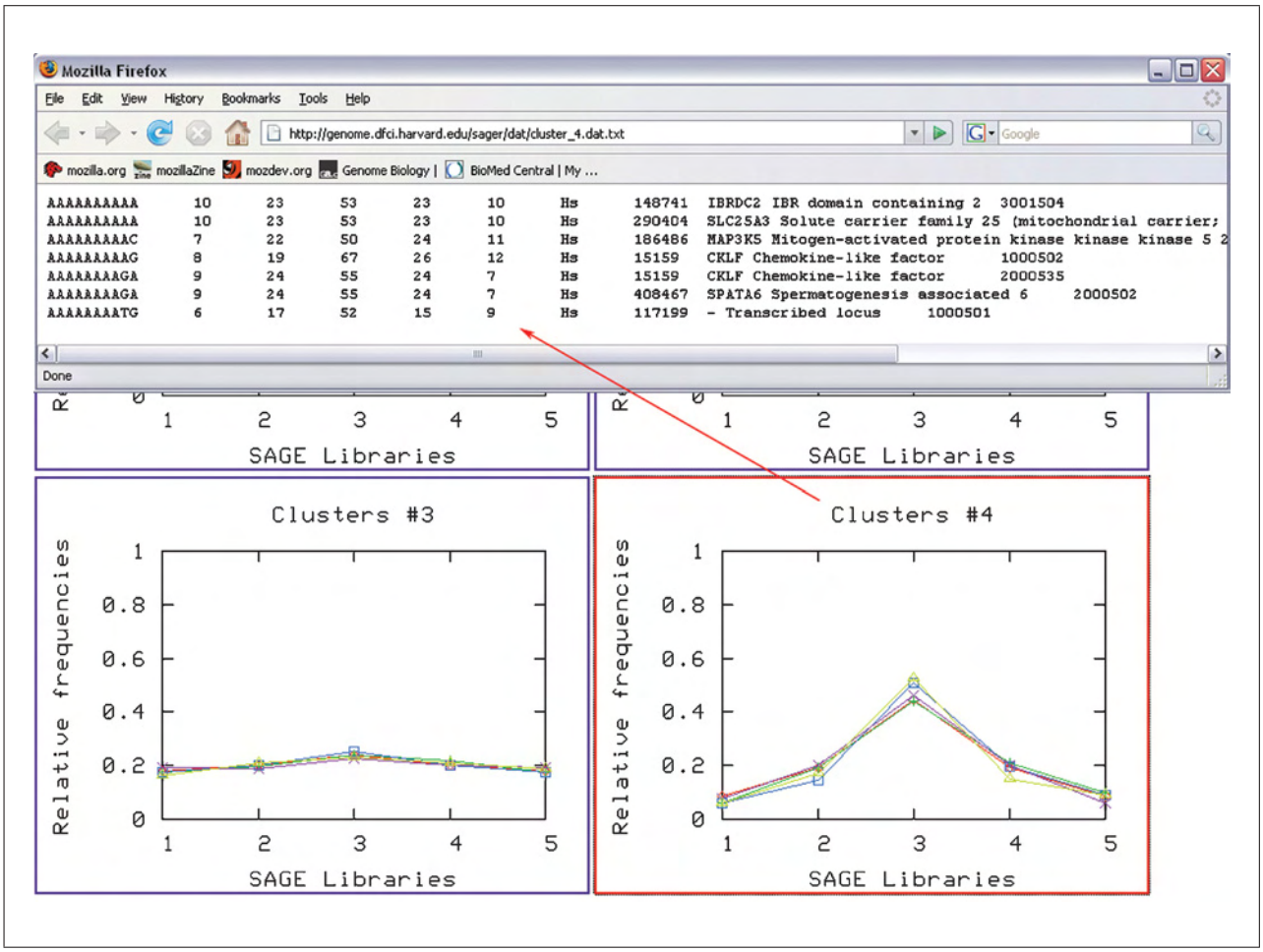
Mozilla Firefox
File Edit View History Bookmarks Tools Help
http://genome.dfci.harvard.edu/sager/dat/cluster_4.dat.txt
mozilla.org mozillaZine mozdev.org Genome Biology | BioMed Central | My ...
AAAAAAAAAA 10 23 53 23 10 Hs 148741 IBRDC2 IBR domain containing 2 3001504
AAAAAAAAAA 10 23 53 23 10 Hs 290404 SLC25A3 Solute carrier family 25 (mitochondrial carrier;
AAAAAAAAAC 7 22 50 24 11 Hs 186486 MAP3K5 Mitogen-activated protein kinase kinase kinase 5 2
AAAAAAAAAG 8 19 67 26 12 Hs 15159 CKLF Chemokine-like factor 1000502
AAAAAAAAGA 9 24 55 24 7 Hs 15159 CKLF Chemokine-like factor 2000535
AAAAAAAAGA 9 24 55 24 7 Hs 408467 SPATA6 Spermatogenesis associated 6 2000502
AAAAAAAATG 6 17 52 15 9 Hs 117199 - Transcribed locus 1000501
Done
SAGE Libraries
SAGE Libraries
Clusters #3
Clusters #4
Relative frequencies
Relative frequencies
SAGE Libraries
SAGE Libraries

Figure 25B.6.9 Screen shot shows cluster #2 and all of its members after clicking on the graph of cluster #2.

Prioritizing data for further analysis

Once particular clusters of interest have been identified, genes can be prioritized for further study based on a variety of criteria. Genes that match specific SAGE tags can be rapidly functionally annotated with Gene Ontology criteria using Web-based programs such as EASE (Hosack et al., 2003), and genes that match a particular function of interest can then be selected. Genes can also be prioritized based on abundance levels or by relative tissue-specificity. It can be very useful to include additional SAGE libraries from public repositories in the analysis to help generate more robust clusters. Some sources of this data include the SAGEmap (*http://www.ncbi.nlm.nih.gov/SAGE/*) and SAGE Genie (*http://cgap.nci.nih.gov/SAGE*) sites found at NCBI and at *http://www.mouseatlas.org*.

Suggestions for improved results

Given the fact that observed SAGE tag levels are actually found in a Poisson distribution about their actual abundance level (Audic and Claverie, 1997), an abundance threshold can be usefully applied to the data prior to submission for cluster analysis. The exact value to use should be determined empirically, and largely depends on how many false positives one is willing to tolerate in each cluster. Tag counts ≥ 5 in at least one of the SAGE libraries is a good value to start with. Significance analysis indicates that when comparing 2 or more libraries, with tag count 5 in one library versus tag count 0 or 1 in the other library, $p \leq 0.05$. This means SAGE tags that are included in clustering analysis are significantly differentially expressed tags.

Using the stand-alone version of the software

To perform the analysis of SAGE data on a desktop computer, obtain a copy of the application from the SAGE Data Analysis Application Web site (*http://genome.dfci.harvard.edu/sager/*) and store it onto the desktop computer, and double click the downloaded file to start installation. Follow instructions to finish the installation process. There will be an application icon called "SAGE Data Analysis" on the desktop. Double click the icon to start the program. The instructions and tutorial of the stand-alone version are included in the software download package. The program is free for public use. This program is distributed in the hope that it will be useful for research purpose, but WITHOUT ANY WARRANTY.

REAGENTS AND SOLUTIONS

Use double-distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

BRS1 primer

5′-Biotin-CCGGGCGCGCCGTAAAACGACGGCCAG$(T)_{19}$-3′

Order HPLC purified from a trusted supplier. The authors recommend using Integrated DNA Technologies (IDT).

BW buffer, 1×

For 2 *stock:*
10 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
1 mM EDTA
2.0 M NaCl
Store up to 1 year at room temperature
Dilute to 1× with H_2O just before use

Linkers

Linker 1A: 5′ TTTGGATTTGCTGGTGCAGTACAACTAGGCTTAATAGGGACATG 3′
Linker 1B: 5′ TCCCTATTAAGCCTAGTTGTACTGCACCAGCAAATCC[amino mod C7] 3′
Linker 2A: 5′ TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG 3′
Linker 2B: 5′ TCCCCGTACATCGTTAGAAGCTTGAATTCGAGCAG[amino mod C7] 3′

The authors recommend using Integrated DNA Technologies for ordering oligonucleotides.

LoTE buffer

3 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
0.2 mM EDTA, pH 7.5 (*APPENDIX 2*)
Store up to 1 year at room temperature

PC8

480 ml phenol, warmed to 65°C
320 ml 0.5 M Tris·Cl, pH 8.0 (*APPENDIX 2*)
640 ml chloroform

Add in sequence and place at 4°C. After 2 to 3 hr, shake again. After an additional 2 to 3 hr, aspirate aqueous layer. Store up to 1 year in aliquots at −20°C or 6 months at 4°C.

Commercially available 1:1 (v/v) phenol/chloroform mix can also be substituted, as long as the pH is preset to 8.0.

PCR primers

Primer 1:	5′ GGATTTGCTGGTGCAGTACA 3′
Primer 2:	5′ CTGCTCGAATTCAAGCTTCT 3′
M13 forward:	5′ GTAAAACGACGGCCAGT 3′
M13 reverse:	5′ GGAAACAGCTATGACCATG 3′

The authors recommend using Integrated DNA Technologies for ordering oligonucleotides.

SAGE PCR buffer, 10×

166 mM ammonium sulfate
670 mM Tris·Cl, pH 8.8 (*APPENDIX 2*)
67 mM $MgCl_2$
100 mM 2-mercaptoethanol
Dispense into aliquots and store up to 1 year at −20°C

SAGE tag–specific primer

5′-GACATGXXXXXXXXXX-(10-bp SAGE tag)-3′

If the SAGE-tag-specific primer has a calculated annealing temperature below 40°C, incorporate additional bases further 5′ on linker 2A (see recipe for linkers) to increase the oligonucleotide melting temperature. The full linker 2A-SAGE tag sequence is as follows:

5′-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATGXXXXXX XXXX-(10-bp SAGE tag)-3′

The SAGE 2000 software has the ability to extract an additional base for an 11-base tag. This may be helpful, as any additional sequence-specific bases may yield a more specific product.

Zeocin-containing low-salt LB plates

For 1 liter:
10 g tryptone
5 g yeast extract
5 g NaCl

Adjust the pH to 7.5 and add 15 g bactoagar. Autoclave solution and allow to cool before adding zeocin to 100 mg/ml.

COMMENTARY

Background Information

Serial analysis of gene expression (SAGE) was first developed in 1995 (Velculescu et al., 1995), and has since been used to generate a large variety of data from normal and cancerous human tissue (Zhang et al., 1997; Boon, et al. 2002), yeast (Velculescu et al., 1997), *C. elegans* (Halaschek-Wiener et al., 2005), *D. melanogaster* (Gorski et al., 2003), mouse (Virlon et al., 1999; Blackshaw et al., 2004), rat (Klimaschewski et al., 2000), and even (with modifications) human oocytes (Neilson et al., 2000).

SAGE is a powerful method for providing genome-wide gene-expression data. In much the same fashion as EST libraries, SAGE utilizes cDNA "tags" which are sequenced and quantified. The 14-bp SAGE tags differ from ESTs essentially by size, allowing subsequent concatenation and high-throughput sequencing in much greater volumes. The location of the anchoring enzyme site is essentially sufficient to uniquely identify the cognate cDNA or gene. The original protocol required relatively large amounts of starting material (2 to 5 μg of polyA mRNA) and was technically quite challenging, frequently giving variable results even in experienced hands. Major improvements were made to the protocol by a number of groups (Datson et al., 1999; Virlon et al., 1999; St. Croix et al., 2000), which collectively gave rise to a version of the protocol known as microSAGE (see Basic Protocol 1), owing to the fact that over 1000-fold less starting material could be readily used for library construction. The critical modifications

appear to have been anchoring the mRNA to magnetic beads prior to cDNA synthesis (rather than after cDNA sythesis via incorporation of a biotinylated oligo(dT) primer as in the original protocol) and optimization of the quantities of reagents used, in particular, the quantities of linkers. Additional improvements, such as heating the ditag concatemers prior to gel purification (Angelastro et al., 1999), have resulted in SAGE libraries with substantially higher insert frequency and larger insert size than in the original protocol. These technical improvements, coupled with the drop in the cost of DNA sequencing, have combined to allow the generation of over 3.5 million human SAGE tags alone, many of which are publicly available for analysis (*http://www.ncbi.nlm.nih.gov/SAGE*).

SAGE analysis has a number of unique advantages over hybridization-based measures of global gene expression, such as microarray analysis (Chapter 22), or approaches such as subtractive hybridization (*UNITS 25B.1 & 25B.2*) and differential display methodologies (*UNITS 25B.3-25B.5*). Since very few mRNAs lack *Nla*III sites, SAGE generates a tag for virtually every cellular mRNA, providing a level of coverage unequaled by any microarray yet available for humans or mice. For these same reasons, SAGE can also serve as a tool for gene discovery and transcript annotation even in species with fully sequenced genomes. The sensitivity of SAGE is limited only by the number of tags that one has the desire or resource to sequence and, with larger numbers of tags sequenced, it becomes possible to determine relatively small (<2-fold) changes in gene expression between samples. Since individual SAGE tag levels are expressed as a percentage of total tags, it is straightforward to compare tag levels among libraries generated by other labs. As more SAGE libraries are generated and made public, these data sets can be used to generate a large-scale atlas of gene expression that is of great use to the whole scientific community. Such a resource is already available for human normal and malignant tissues at NCBI (*http://www.ncbi.nlm.nih.gov/SAGE*), and libraries from other species are available from various sources (see Internet Resources for a partial list).

SAGE data have been poorly exploited by clustering analysis owing to the lack of appropriate statistical methods that consider their specific properties. In the analysis methods proposed in Basic Protocol 3, SAGE data were modeled by Poisson statistics, and the Poisson-based distances were implemented into the K-means procedure in clustering SAGE data. It has been demonstrated that the Poisson-based distances have advantages over the Pearson correlation and Euclidean distance in clustering SAGE data (Cai, et al., 2004). These commonly used distance measurements, e.g., Pearson correlation and Euclidean, in microarray data analysis were shown not to be suitable for SAGE data analysis. The poor performance of Pearson correlation and Euclidean distance in SAGE data analysis may be due to the fact that the Pearson correlation distance only uses the shape of the curves, but neglects the magnitude of changes, while the Euclidean distance takes the difference between data points directly and may be overly sensitive to the magnitude of changes.

The main drawbacks of SAGE analysis are the time and expense required to generate sufficient numbers of tags to examine expression of low and moderate-abundance mRNAs. The price of sequencing has dropped considerably in the past few years, but real costs still remain around $0.25/tag. For researchers simply hoping to identify a handful of differentially expressed genes in their sample of interest, subtractive hybridization (*UNIT 25B.1*), differential display methodologies (*UNITS 25B.3-25B.5*), or even the use of commercially available microarray technology may prove more cost-effective. An additional drawback of SAGE is the requirement that a large body of cDNA/EST sequence must be available from the organism being studied in order to match SAGE tags to the specific mRNAs. This effectively limits the use of SAGE to model organisms. Another drawback of the method is the occasional failure of a SAGE tag to match a predicted gene or to be long enough to easily isolate a full-length cDNA clone. While this happens at relatively low frequency for high abundance transcripts in model organisms, it can limit the interpretation of the data in some cases.

As a result, several approaches, most of which are variations of conventional RT-PCR, have been developed to identify these unknown or anonymous SAGE tags. There has been marked improvements in strategies used to identify unknown SAGE tags by reverse-cloning cDNA fragments, collectively called reverse SAGE (rSAGE; see Basic Protocol 2). First, the cloning process is similar to the original SAGE protocol; therefore, only cDNA pieces which are 3′ to the most 3′ anchoring enzyme site are used as templates for subsequent PCR amplification and subcloning

(Polyak et al., 1997; also see Internet Resources, SAGEnet). Second, the use of betaine allows for a prolonged PCR touchdown that results in more specific priming.

Critical Parameters and Troubleshooting

MicroSAGE

The two key determinants of a successful SAGE library are quantity and purity of ditags. To ensure obtaining many ditags, carefully optimize the starting reaction and scale up the number of PCR reactions as desired. For certain low-yield preparations, the authors have gone as high as 700 PCR reactions of 50 μl to generate the starting material. For purity, ensure that the 102-bp and the 80-bp bands are well separated, and be very careful not to extract any of the 80-bp band. Run the gel as long as possible and do not overload the wells (no more than 10 μl per well, despite the large number of gels this will require). Do the same for the 26-bp cut ditag band (avoiding the 40-bp linker band).

One other problem that has been encountered occasionally is contamination of reagents following construction of libraries, which will result in 102-bp bands in the no-ligase control in the initial optimization PCR reactions. To avoid this, be very careful to avoid splashes and not reuse tips during the scale-up or initial purification of the 102-bp band. Make separate aliquots of LoTE buffer, PC8, ammonium acetate, and ethanol for each library during these steps to reduce the likelihood of contamination. Use aerosol-barrier tips wherever possible.

A final common cause of experimental failure is low-quality reagents. Wherever possible, order supplies from the sources specified in the protocol. The authors have most frequently observed problems with the *Nla*III enzyme and the linkers. Always store *Nla*III in aliquots at −80°C, do not reuse aliquots, and try to have the enzyme shipped on dry ice if possible. The authors order linkers prekinased, but always check via self-ligation to ensure that a sufficiently large fraction of the linkers is properly phosphorylated.

rSAGE

For the rSAGE procedure, much depends on the quality of RNA used in the sample. It would be best to use the same batch of RNA that was originally used to construct the SAGE library. As most interesting SAGE tags are those that are expressed in abundance in one RNA sample and not in another, it is advisable to make a reverse-SAGE library of such a control tissue. It is not uncommon to generate multiple PCR bands from a tag-specific rSAGE amplification. Identifying a PCR product that is specific to the experimental rSAGE library and not present (or less apparent) in the control would help in the cloning and identification process.

The most technically challenging aspect of reverse-cloning SAGE tags is the PCR of a specific cDNA with the tag-specific primer. The rSAGE-amplified library used as a template for this PCR reaction consists solely of 3′-cDNA ends which have the linker2-SAGE tag on the 5′ end and a oligo dT-M13 forward sequence on the 3′ end. The PCR of a specific product is difficult when the reverse primer (M13 Forward) anneals to all templates, and the forward primer (SAGE-tag specific) shares the same sequences on the 5′ end. Specificity is conferred only by the last 10 bases on the forward primer, representing the unique 10-base SAGE tag. One may also choose to incorporate an additional SAGE-tag base, information that the SAGE 2000 software can extract from the raw data. The SAGE tag–specific PCR is executed with a prolonged touchdown using an automatic hot-start *Taq* polymerase (i.e., Platinum *Taq*; Invitrogen). As a 15-cycle touchdown requires 46 denaturing cycles, betaine is used as a *Taq* polymerase protectant. The authors strongly advise against switching to a proofreading DNA polymerase, such as *Pfu* or Vent, in the PCR reactions. Proofreading enzymes have significant 3′-5′ exonuclease activity which may digest the 3′ end of the SAGE tag–specific primer. Even one-base differences may reduce the specificity of the PCR product.

Designing of SAGE tag–specific primers is a matter of much debate. Only the 3′-most ten bases of the oligonucleotide contains tag-specific sequences, and the rest of the primer at the 5′ end consists of linker sequences which are shared by all the cDNAs in the amplified rSAGE library. As a result, the authors empirically use CACATG-XXXXXXXXXX as a guideline for primer design where the Xs refer to the specific sequence in the SAGE tag of interest. Only six bases are nonspecific, and the relatively low annealing temperatures allow for an extended touchdown starting at a temperature that is well above the oligonucleotide melting point. However, if the rSAGE-specific primer has an annealing temperature which is too low, there is a risk of the primers melting off the template before the extension cycle. Therefore, if the calculated T_m of the SAGE tag specific primer is below 40°C, it is advisable

Table 25B.6.1 Troubleshooting for SAGE Reactions

Problem	Possible Cause	Solution
MicroSAGE		
No PCR product with control primers following cDNA synthesis	Dynabeads inactive	Store Dynabeads at 4°C only; do not freeze
	Reverse transcriptase inactive	Replace reverse transcriptase
	RNA degraded prior to homogenization	Minimize delay between tissue harvesting and homogenization
	Cells insufficiently lysed	Homogenize tissue thoroughly. Use homogenization by Polytron only.
Ditag PCR product is slightly shorter (running at ~90 bp) and will not redigest with *Nla*III	Failure to completely remove *E. coli* DNA polymerase I following second strand cDNA synthesis	Do not omit or shorten SDS washes or 75°C heat inactivation step
PCR product in no-ligase control at 100 bp	Contamination of reagent by ditags from a previously constructed SAGE library	Use separate aliquots of LoTE buffer, ammonium acetate, and PC8 for each large-scale ditag purification. Use aerosol pipet tips.
Ditag yield low (100-bp band <80-bp band)	Ratio of linkers to cDNA too high	Reduce amount of linkers in ligation
	cDNA synthesis inefficient	See advice in steps 1-11
	PCR conditions not optimized	Titrate dNTP concentration and number of amplification cycles
	Quantity of starting material too low	Increase amount of starting material
Ditags do not cut with *Nla*III following purification	*Nla*III inactive	Store enzyme in aliquots at −80°C. Do not reuse thawed aliquots.
	Ditags insufficiently pure	Run Qiaquick gel extraction on eluate (see step 44)
Ditag concatemers not generated efficiently	Insufficient quantity of purified ditags used in ligation	Increase quantity of cDNA used for large-scale ditag prep and/or increase number of cycles of amplification
	Ditags used in ligation are insufficiently pure	Run preparative gel longer to more efficiently separate 100- and 80-bp bands
Ditag concatemers most concentrated at high molecular weight (3kb) and do not clone efficiently	Concatemer ligation not heated properly	Heat at 65°C and chill on ice immediately

continued

Table 25B.6.1 Troubleshooting for SAGE Reactions, *continued*

Problem	Possible Cause	Solution
Concatemers have a high (5%) frequency of duplicate ditags	Too many cycles of PCR used to reamplify ditags	Reduce number of cycles used. Increase amount of starting material when making cDNA
rSAGE procedure		
No SAGE tag-specific PCR product	Degraded RNA	Use fersh RNA
	Error in generation of rSAGE amplified library	Reconstruct rSAGE library
	Poor tag specific primer design	Redesign primer (see Critical Parameters and Troubleshooting)
	Low abundance transcript	Increase the number of amplification cycles
Multiple SAGE tag–specific PCR products	Multiple splice variants or multiple gene identities for a given SAGE tag	Clone all PCR amplicons
		Construct a control rSAGE library and select amplicons not present in control library
	Nonspecific priming	Start the PCR touchdown cycles at a higher temperature
Poor sequence quality	Use of M13 forward primer for cycle sequencing	Use another universal primer on the pCR vector for sequencing—e.g., M13 reverse, T3, T7

to incorporate more of the linker sequence to raise the melting temperature of the oligo.

In the rare case that the SAGE tag in question lies immediately 5′ to the polyA tail, reverse-SAGE may yield no additional information, and the PCR product may be too small to adequately visualize on a 1.5% agarose gel.

Additional troubleshooting guidelines are presented in Table 25B.6.1.

Anticipated Results

If Basic Protocol 1 is followed closely, libraries containing >85% inserts with an average size of 30 to 50 tags (450 to 750 bp) should be routinely generated. This should enable one to obtain a SAGE data set of 50,000 tags after ~2000 individual sequencing reactions.

If the above guidelines for rSAGE (see Basic Protocol 2) are followed, one should be able to clone the cDNA, usually 75 to 400 bp, from which a given SAGE tag is generated. This cDNA fragment would stretch from the 3′-most anchoring-enzyme site to the poly(A) tail. The additional sequence data can be used to BLAST genome databases (*UNIT 19.3*) or be used to generate primers for 5′ RACE (*UNIT 15.6*). The cloned fragment may also be used for northern analyses (*UNIT 4.9*) or in situ hybridizations (*UNIT 14.3*).

Time Considerations

MicroSAGE

The time typically taken for RNA preparation through *Bsm*FI digestion is 10 to 14 hr. Blunt-ending and ditag-ditag ligation take 2 to 3 hr. Ditag amplification and PCR optimization take 2 to 3 hr and large-scale ditag amplification and purification take 6 to 8 hr/day for 2 days. Ditag digestion and purification take 6 to 8 hr. Concatemer formation, purification, and subcloning take 6 to 8 hr. Template cleanup and transformation take 4 to 6 hr. PCR of library clones and gel analysis take 4 to 5 hr.

If a high-quality SAGE library is produced, it will require ~2000 sequencing reactions to obtain 50,000 tags. This will take anywhere

from an additional 1 week to 3 months, depending on the resources and sequencing capacity.

rSAGE

Generating purified double-stranded cDNA typically takes 4.5 hr. Cleaving the cDNA with the anchoring enzyme (*Nla*III), magnetic bead purification, ligating linkers to cDNA, and release of 3′ cDNA fragments from magnetic beads with *Asc*I typically takes 6 to 8 hr. PCR generation of amplified rSAGE libraries takes 2.5 to 3.5 hr. SAGE tag-specific PCR takes 3.5 to 4.5 hr. TOPO-TA cloning and subsequent sequencing is user-dependent.

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Internet Resources

http://www.sagenet.org

SAGEnet. Contains instructions for obtaining SAGE analysis software, downloadable SAGE libraries from human, mouse and yeast, and a comprehensive bibliography of SAGE papers.

http://www.ncbi.nlm.nih.gov/SAGE

Serial analysis of gene expression at NCBI.

http://www.ncbi.nlm.nih.gov/CGAP

Cancer Genome Anatomy project. Contains full downloadable predicted tag data for human, mouse, rat, zebrafish, and cow. Also contains a large number of downloadable human SAGE libraries (containing >3.5 million total tags), as well as tools for submitting SAGE data for public access and tools for searching tag abundance levels in the publicly available human SAGE data.

http://www.umich.edu/~ehm/eSAGE

eSAGE at University of Michigan. Helpful software for SAGE data analysis.

http://www.invitrogen.com

iSAGE at Invitrogen. Integrated kit and software package for conducting microSAGE. The protocol used is very similar to the one described here.

http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/mergesagetags.html

Merge SAGE tags at Harvard Medical School. Helpful tool for merging SAGE data files and downloaded predicted tag identify files (from NCBI).

APPENDIX: ALGORITHM FOR POISSON-BASED SIGNIFICANCE ANALYSIS

In a SAGE experiment, a set of transcripts from a cell or tissue is sampled for tag extraction. Considering the numerous types of transcripts present in a cell or tissue and the small probability of sampling a particular type of transcript, the authors assume that the number of sampled transcripts of each type is approximately Poisson distributed. Statistically, when this actual sampling process is random enough, Poisson would be the most practical and reasonable assumption compared to other probability models. This assumption leads to the following probability models used for significance analysis and clustering analysis of SAGE data.

Based on Poisson assumption, the authors developed a significance analysis algorithm ("SA algorithm") to detect differentially expressed tags in SAGE data. The input to the SA algorithm is a tab-delimited file containing multiple sage libraries. The SA algorithm can simultaneously compare two or more SAGE libraries. The output of SA algorithm is a set of P values of tests for the significance of the difference in gene expression. Genes with significantly small P values are identified as differentially expressed across different libraries. The P values are calculated in the following way:

Letting X_{ij} be the number of copies of tag i in library j, three sums are defined:

$$M_{i.} = \sum_j X_{ij}; \text{ The sum of counts of tag } i \text{ over all libraries.}$$

$$M_{.j} = \sum_i X_{ij}; \text{ The sum of tag counts over all tags in library } j.$$

$$M = \sum_{i,j} X_{ij}; \text{ The sum of tag counts over all tags and libraries.}$$

Under the null hypothesis that there is no expression difference across libraries, $M_i M_j / M$ copies are then expected to be observed for tag i in library j. Further, considering that the tags are extracted from a random sample of transcripts in cell, it is reasonable to assume X_{ij} is Poisson distributed with means $\lambda_{ij} = M_i M_j / M$.

The χ^2 statistic is used to test the deviation of observed counts from expected counts:

$$TS_i = \sum_{j=1}^{k} \frac{(X_{ij} - \lambda_{ij})^2}{\lambda_{ij}}$$

where k is the number of libraries compared.

When k is large or λ_{ij} is not small (<5), TS_i is approximately χ^2 distributed with degree of freedom of $k-1$ (χ^2_{k-1}), the SA algorithm calculates the P values using the approximated χ^2_{k-1}. However, when k and λ_{ij} are small, there is a large departure of TS_i from χ^2_{k-1}, the SA algorithm calculates exact P value of observed TS_i based on the Poisson distribution of X_{ij}.

Representational Difference Analysis

This unit provides a protocol for performing representational difference analysis (RDA); a technique that couples subtractive hybridization to PCR-mediated kinetic enrichment for the detection of differences between two complex genomes. RDA requires the generation of representations from two pools of nearly identical DNA varying only in polymorphisms, deletions/amplifications, rearrangements, or exogenous pathogens. A representation or subset of the genome is used rather than the entire genome, since the full complexity of genomic DNA is unfavorable for hybridization to proceed to completion. In its original formulation by Lisitsyn and colleagues (1993), 2% to 15% of the genome is included in the representation, the percentage being dependent on the frequency of restriction endonuclease sites and the efficiency of PCR amplification of these restriction-generated fragments. While RDA was first developed for genomic DNA, subsequent modifications have been devised to look for differences in transcript expression.

RDA starts with the digestion of two comparison samples of DNA (see Basic Protocol 1) or cDNA (see Basic Protocol 2) with a frequently cutting restriction enzyme. Some consideration should be given to which of the two genomes is designated tester and which is designated driver. In principle, the tester should contain DNA restriction fragments not found in the driver. Specific linkers are ligated to DNA restriction fragments from each pool and amplicons are generated by PCR. Linkers are then removed from both samples and a new linker is added only to size-selected tester amplicons. These tester amplicons are mixed and melted with a large excess of driver amplicons lacking linkers. Hybridization between complementary single strands is allowed to proceed, resulting in the generation of three species of double-stranded DNA fragments: (1) both strands derived from driver DNA (lacking linkers on either strand), (2) hybrids with one strand from driver (no linker) and one from tester (with linker), and (3) both strands from tester DNA (linkers on both strands). Excess driver will soak up DNA fragments common to both samples (i.e., tester:driver), and only the DNA fragments unique to the tester (i.e., the tester:tester population) will be exponentially amplified and kinetically enriched when linker-specific primers are used. Iterative rounds of subtractive/kinetic enrichment against driver amplicons is performed until distinct difference products can be cloned.

GENOMIC REPRESENTATIONAL DIFFERENCE ANALYSIS

BASIC PROTOCOL 1

This protocol describes RDA for genomic DNA derived from tissues or cells. Modifications for performing cDNA RDA are discussed below (see Basic Protocol 2).

Materials

Tester and driver DNA samples
Phenol (Amresco; *UNIT 2.1A*)
Phenol:chloroform:isoamyl alcohol (Amresco; *UNIT 2.1A*)
20 µg/µl glycogen
TE buffer, pH 8.0 (*APPENDIX 2*)
Primers/oligomers, HPLC purified (Table 25B.7.1)
400 U/µl T4 DNA ligase and 10× buffer (New England BioLabs; *UNIT 3.14*)
5× RDA PCR buffer (see recipe)
dNTP chase solution: 4 mM (each) dGTP, dATP, dTTP, dCTP; store at −20°C
5 U/µl *Taq* DNA polymerase (Invitrogen; *UNIT 3.5*)
Mineral oil
Isopropanol
10 M ammonium acetate (*APPENDIX 2*)

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Table 25B.7.1 Prototypic Primers Used in RDA

Primer Type	Name[a]	Sequence[b]
Representation		
24-mers	R*Bgl*24	5′-**AGCACTCTCCAGCCTCTCACCG**CA-3′
	R*Bam*24	5′-**AGCACTCTCCAGCCTCTCACCG**AG-3′
	R*Hin*d24	5′-**AGCACTCTCCAGCCTCTCACCG**CA-3′
	RXxx24	5′-**AGCACTCTCCAGCCTCTCACCG**xx-3′
12-mers	R*Bgl*12	5′-GATCT**GCGGTGA**-3′
	R*Bam*12	5′-GATCC**TCGGTGA**-3′
	R*Hind*12	5′-AGCTT**GCGGTGA**-3′
	RXxx24	5′-xxxxxx **CGGTGA**-3′
Odd cycle		
24-mers	O*Bgl*24	5′-**ACCGACGTCGACTATCCATGAAC**A-3′
	O*Bam*24	5′-**ACCGACGTCGACTATCCATGAAC**G-3′
	O*Hind*24	5′-**ACCGACGTCGACTATCCATGAAC**A-3′
	OXxx24	5′-**ACCGACGTCGACTATCCATGAAC**x-3′
12-mers	O*Bgl*12	5′-GATCT**GTTCATG**-3′
	O*Bam*12	5′-GATCC**GTTCATG**-3′
	O*Hind*12	5′-AGCTT**GTTCATG**-3′
	OXxx24	5′-xxxxx **GTTCATG**-3′
Even cycle		
24-mers	E*Bgl*24	5′-**AGGCAACTGTGCTATCCGAGGG**AA-3′
	E*Bam*24	5′-**AGGCAACTGTGCTATCCGAGGG**AG-3′
	E*Hin*d24	5′-**AGGCAGCTGTGGTATCGAGGGA**GA-3′
	EXxx24	5′-**AGGCAACTGTGCTATCCGAGGG**Ax-3′
12-mers	E*Bgl*12	5′-GATCT**TCCCTCG**-3′
	E*Bam*12	5′-GATCC**TCCCTCG**-3′
	E*Hin*d12	5′-AGCTT**CTCCCTC**-3′
	EXxx12	5′-xxxxx **TCCCTCG**-3′

[a]R primers are used only in making representations of the tester and driver DNAs. The O and E primers are used in odd and even iterations of the subtractive/enrichment process. These were previously designated J and N in the original protocol (Lisitsyn et al., 1993).

[b]Underscores indicate restriction sites that are variable, but limited to those comprising restriction sites (i.e., can be changed to accommodate other enzymes). Nucleotides shown in bold outline invariant core sequences of the primers. Nucleotides which are neither bold nor underscored are completely variable.

100% ethanol, ice cold
70% ethanol, room temperature
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
EE × 3 hybridization buffer (see recipe)
5 M NaCl
5 µg/µl glycogen in TE buffer (see *APPENDIX 2* for TE buffer)
10 U/µl mung bean nuclease and 10× buffer (New England BioLabs; *UNIT 3.12*)
50 mM Tris·Cl, pH 8.9 (*APPENDIX 2*)

Thermal Cycler (Perkin-Elmer Model 480 preferred)
24-mm GF/C glass microfibre filters (Whatman)
Dialysis tubing, 6,000 to 8,000 MWCO (Spectra/Pore)
Flat blunt forceps
18-G needle

Additional reagents and equipment for restriction digestion (*UNIT 3.1*), agarose gel electrophoresis (*UNIT 2.5A*), ethanol and isopropanol precipitation (*UNIT 2.1A*), and quantifying DNA by absorbance spectroscopy (*APPENDIX 3D*), gel isolation (*UNIT 2.6*), and sequencing (*UNIT 7.1*).

NOTE: Use de-ionized, distilled water in all recipes and protocol steps, and ensure that the water is RNase/DNase free. Since minute amounts of contaminating DNA may be detected by RDA, use barrier pipet tips throughout the protocol.

Prepare amplicons and representation: Enzyme restriction of tester and driver DNA

1. Using 10 U enzyme per microgram DNA and a total volume of 200 µl (each), separately digest 5 µg tester and driver DNA samples with the restriction enzyme chosen for representation (*UNIT 3.1*). Analyze 40 µl (1 µg) of each reaction by electrophoresis on a 1% agarose gel (*UNIT 2.5A*) to confirm complete digestion. Bring volume of remaining digest to 400 µl each with water.

   This step provides three to four times the DNA needed for the preparation of amplicons; therefore, if the amount of starting DNA is a limiting factor, as little as 1 to 2 µg DNA can be used.

   BglII, BamHI, and HindIII are the enzymes which were used in the original RDA publication by Lisitsyn and colleagues (1993). Oligomer/primers compatible with each of these enzymes are listed in Table 25B.7.1. These enzymes with corresponding oligomers have been extensively and successfully used in RDA applications; however, other enzymes may be used by adapting the restriction sites adjacent to the core sequences. In particular, four-base cutters may be more appropriate for less complex genomes.

2. Extract digested tester and driver with 1 vol phenol (400 µl each) followed by 1 vol phenol:chloroform:isoamyl alcohol (400 µl each). Ethanol precipitate DNA (*UNIT 2.1A*), adding 20 µg glycogen and microcentrifuging at 4°C to increase recovery. Dry pellets and resuspend at 0.1 µg/µl in TE buffer, pH 8.0, instead of water in the final step. Confirm DNA concentration by comparison to dilution of known standards by agarose gel electrophoresis (*UNIT 2.5A*).

3. Resuspend HPLC-purified primers/oligomers in water at 62 pmol/µl, an OD_{260} of 6 or 12 AU/ml for 12- and 24-mers, respectively (*APPENDIX 3D*).

   HPLC purification of oligomers is critical for minimizing false positive RDA bands (O'Neill and Sinclair, 1997).

Ligate adapters onto driver and tester DNA

4. Mix the following in thermal cycler tubes colored differently for tester and driver DNA:

   2 µl water
   3 µl 10× ligase buffer
   7.5 µl 12-mer (R primer)
   7.5 µl 24-mer (R primer)
   10.0 µl (1 µg) driver or tester DNA digest
   30 µl total volume.

   The use of tubes of different colors throughout the protocol helps distinguish between driver and tester samples to avoid confusion and cross-contamination of DNA.

5. Place tubes in a thermal cycler at 55°C. Program the thermal cycler to decrease the temperature to 4°C over 1 hr.

   Slow annealing allows the 12- and 24-mers to form a temporary bridging complex with cohesive ends complementary to the restriction sites on the ends of the digested DNAs.

   The Perkin-Elmer Model 480 is preferred because of its larger tube capacity, but any 96-well thermal cycler may also be used.

6. Add 1 µl of 400 U/µl T4 DNA ligase, mix by gentle pipetting, and incubate 12 to 16 hr at 14°C.

   This step results in ligation of the 24-mers onto the 5′ ends of the DNAs. The temperature used is below the T_m of the four base duplexes formed by the overhanging ends.

7. Transfer ligation product to 1.5-ml microcentrifuge tubes matching the colors used above (step 4). Dilute adapter-ligated tester and driver DNA to 1 ng/µl by adding 970 µl TE buffer.

PCR-amplify driver and tester amplicons

8. Prepare two tubes of PCR mix for preparation of tester amplicon and twelve tubes for driver amplicon, each containing:

   240 µl water
   80 µl 5× RDA PCR buffer
   32 µl dNTP Chase solution
   8 µl 24-mer oligonucleotide (R primer)
   360 µl total volume.

9. Add 40 µl diluted adapter-ligated tester or driver DNA (40 ng) to corresponding PCR tubes (two for tester and twelve for driver) and place tubes in a thermal cycler 1 to 2 min at 72°C.

10. Fill-in 3′-recessed ends of the ligated adapters by adding 3 µl of 5 U/µl (15 U) *Taq* DNA polymerase to each tube, mix by pipetting, and overlay with 110 µl mineral oil. Incubate 5 min at 72°C.

    If using a 96-well PCR machine, double the number of tubes and halve the amount of PCR mixture for each tube such that four tubes of tester and twenty-four tubes of driver amplicon are made. With the 96-well PCR machine, no mineral oil is required. Do not let the tubes cool below 72°C in steps 9 or 10.

11. Perform the following two-step PCR program:

20 cycles:	1 min	95°C	(denaturation)
	3 min	72°C	(extension)
Final step:	10 min	72°C	(extension).

Quantitate amplicons and remove linkers

12. Pipet off as much mineral oil as possible. Combine the contents of both tester PCR tubes into a single 1.5-ml microcentrifuge tube. Combine driver tubes pairwise into single microcentrifuge tubes (i.e., six driver tubes total).

    For the 96-well PCR format, combine the contents of four PCR tubes into a single 1.5-ml microcentrifuge tube.

13. Extract each tube with 1 vol phenol followed by 1 vol phenol:chloroform:isoamyl alcohol, isopropanol precipitate with 20 µg glycogen, and dry the pellets (*UNIT 2.1A*).

14. Resuspend driver and tester amplicons in TE buffer at a concentration between 0.2 to 0.4 µg/µl (expecting ~15 µg of DNA from each 0.5-ml PCR tube). Pool driver DNA into a single tube. Confirm concentrations of driver and tester DNA by agarose gel electrophoresis (*UNIT 2.5A*) against DNA standards.

    Enough of the driver amplicon needs to be prepared to provide sufficient amounts of DNA such that all rounds of hybridization use aliquots that are identical and derived from the same source. Calculate the total amount of driver DNA needed for the experiment (~40 µg/round) and if necessary, scale up driver amplicon production or perform additional driver amplicon amplifications and pool.

15. Digest 150 µg driver DNA and 15 µg tester DNA with initially chosen restriction endonuclease (step 1) in volumes of 400 µl to remove the adapters.
16. Repeat step 2 and resuspend in 125 µl TE buffer.

    Expect the concentration of tester to be ~0.1 µg/µl and that of driver to be ~1 µg/µl.
17. Dilute 2 µl resuspended driver amplicon digest with 18 µl water to an expected concentration of 0.1 µg/µl. Load 0.2, 0.4, and 0.6 µg driver and tester amplicon digests and compare with DNA standards by 2% agarose gel electrophoresis (*UNIT 2.5A*). Using electrophoresis results as a guide, perform final dilution with TE buffer such that the driver amplicon digest concentration is 0.5 µg/µl and the tester amplicon digest concentration is 50 ng/µl.

Change adapters on tester amplicon

18. Load 5 µg (100 µl) tester amplicon DNA digest on a 1% agarose gel (*UNIT 2.5A*). Electrophorese at appropriate voltage until DNA in the range from 150 to 1500 bp can be resolved.
19. With a clean razor blade, cut two full thickness slits in the running lanes, one at 150 and another at 1500 bp.
20. Soak small pieces of 24-mm GF/C glass microfibre filter and 6,000- to 8,000-MWCO dialysis tubing in water. Make a two-layer barrier of filter and dialysis tubing and cut into rectangles slightly higher and wider than the agarose lane. Using a blunt flat forceps, insert the filter/dialysis tubing barrier into each of the slits with the filters facing the loading wells.

    Be sure that the entire running lane is blocked by both the filter as well as the dialysis tubing.
21. Resume electrophoresis until DNA between 150 and 1500 bp has migrated onto the filter/dialysis tubing. Stop the electrophoresis and carefully remove the DNA embedded filter/dialysis tubing from the 150-bp slit.

    DNA larger than 1500 bp should be blocked from migrating past the filter/dialysis membrane in the 1500-bp slit.

    In the author's hands, this method gives better recovery than gel isolation and elution.
22. Cut the lid off a 0.5-ml PCR tube and puncture a hole in the bottom with an 18-G needle so that DNA can elute. Make a collecting apparatus comprised of the PCR tube placed inside a 1.5-ml microcentrifuge tube.
23. Place the filter/dialysis membrane into the PCR tube of the collecting apparatus. Microcentrifuge the collecting apparatus 5 min at 8,000 rpm, room temperature.
24. Discard the PCR tube and filter/dialysis tubing. Bring up volume of collected liquid to 400 µl with water and extract with 1 vol phenol followed by 1 vol phenol:chloroform:isoamyl alcohol. Ethanol precipitate DNA with 20 µg glycogen and dry the pellet as described (step 2).
25. Dissolve the DNA pellet in 30 µl TE buffer, check DNA concentration by agarose gel electrophoresis against DNA standards (*UNIT 2.5A*), and adjust the concentration to 0.1 µg/µl.
26. Ligate 1 µg purified tester amplicon DNA digest to primer set O, as described in steps 4 to 6 above.

    The R set of primers used to make the driver amplicons is never used in subsequent subtractive/kinetic enrichment rounds to prevent driver amplification as a result of uncleaved primers.

27. Dilute the pellet to a concentration of 10 ng/µl by adding 70 µl TE buffer

If using HindIII, dilute the pellet to 25 ng/µl by adding 10 µl TE buffer.

Perform subtractive/kinetic enrichment

28. In a microcentrifuge tube, combine driver and tester by mixing 80 µl driver amplicon DNA digest (0.5 µg/µl) and 40 µl diluted tester amplicon ligate (0.4 µg for representations made with most six cutters or 1 µg for *Hin*dIII representation).

The first hybridization is done at a tester:driver ratio of ~1:100.

29. Extract once with 120 µl phenol:chloroform:isoamyl alcohol.

30. Ethanol precipitate DNA with ammonium acetate as follows:

    a. Add 30 µl of 10 M ammonium acetate and mix by pipetting.
    b. Add 300 µl (2 vol) ice-cold 100% ethanol.
    c. Add 1 µl (20 µg) glycogen and mix by inverting.
    d. Chill 10 min at −70°C.
    e. Microcentrifuge 20 min at 13,000 rpm, room temperature.
    f. Carefully remove the supernatant.
    g. Wash the pellet with 1 ml room-temperature 70% ethanol.
    h. Dry pellet.

31. Add 4 µl EE × 3 hybridization buffer to the pellet. Resuspend by pipetting, incubate 5 min at 37°C, vortex 2 min, and then microcentrifuge at maximum speed to collect the sample at the bottom.

32. Transfer resuspended DNA to a PCR tube. In another PCR tube, add 1 µl of 5 M NaCl. Place both tubes in a thermal cycler preheated to 95°C and incubate 1 min. Centrifuge the tubes briefly to collect the contents at the bottom and immediately transfer the denatured DNA to the tube containing NaCl. Mix well by pipetting and overlay with 35 µl mineral oil.

33. Incubate the tube containing DNA and NaCl in the thermal cycler for an additional 4 min at 95°C to ensure that all DNA species are denatured.

34. Set the thermal cycler to hold >20 hr at 67°C. Incubate at least 18, but not more than 48 hr, to allow the DNAs to hybridize to complementary strands.

As a result of the vast excess of driver, the majority of fragments common to both the driver and tester populations will rapidly form driver:driver or tester:driver complexes. The fragments unique (or at a relatively higher quantity) in the tester will require a significantly longer period of time to completely hybridize and form tester:tester complexes.

Perform selective amplification

35. Remove as much of the mineral oil as possible without losing the hybridizing mixture. Dilute the DNA stepwise to a concentration of 0.1 µg/µl by first adding 8 µl of 5 µg/µl glycogen in TE buffer and mixing by pipetting, then adding 23 µl TE buffer and again mixing by pipetting, and finally adding 364 µl TE buffer and vortexing.

36. To fill-in the adapter ends, make two tubes of PCR mix (not containing 24-mer):

    235 µl water
    80 µl 5× RDA PCR buffer
    32 µl dNTP chase solution
    347 µl total volume.

Add 40 µl diluted hybridized DNA (4 µg) to each tube. Place tubes in thermal cycler set at 72°C.

This reforms priming sites at both ends of tester:tester complexes necessary for exponential amplification of difference products.

37. Add 3 µl *Taq* DNA polymerase, mix by pipetting, and incubate an additional 5 min.

38. Add 10 µl 24-mer primer (O primer set), mix by pipetting, and overlay with mineral oil.

    If using a 96-well thermal cycler, double the number of tubes and halve the PCR recipe in each tube. In this case, addition of mineral oil is not necessary.

39. Perform the following two-step PCR program:

10 cycles:	1 min	95°C	(denaturation)
	3 min	72°C	(extension)
Final step:	10 min	72°C	(extension).

    For the OBgl 24 primer, a lower annealing temperature of 70°C is required.

40. Remove as much mineral oil as possible and combine the contents of the PCR tubes in a microcentrifuge tube. Extract and isopropanol precipitate as described (step 13), but dissolve the pellet in 40 µl water and do not pool DNA.

41. Digest single-stranded templates with mung bean nuclease (MBN) by mixing:

    14 µl water
    4 µl 10× mung bean nuclease buffer
    20 µl amplified difference product
    2 µl 10 U/µl mung bean nuclease (MBN)
    40 µl total volume.

    Incubate at 30°C for 30 min.

42. Add 160 µl of 50 mM Tris·Cl, pH 8.9. Inactivate MBN by incubating 5 min at 98°C.

43. Prepare two tubes of PCR mix (360 µl) containing the O 24-mer primer as in step 8. Add 40 µl MBN-treated difference product in each tube and place in a thermal cycler set at 72°C.

    For OBgl 24-mer use an annealing temperature of 70°C.

44. Add 3 µl of 5 U/µl (15 U) *Taq* DNA polymerase to each tube, mix by pipetting, overlay with 110 µl mineral oil, and incubate 5 min at 72°C.

    Again, double the number of PCR tubes and halve the given recipe placed in each tube if using a 96-well PCR machine.

45. Perform the following two-step PCR program:

20 cycles:	1 min	95°C	(denaturation)
	3 min	72°C	(extension)
Final step:	10 min	72°C	(extension).

    For the OBgl 24 primer, a lower annealing temperature of 70°C is required.

46. Run 10 µl amplified product on a 2% agarose gel with DNA concentration standards (*UNIT 2.5A*). If necessary to improve the yield, perform 1 to 3 more cycles after addition of 3 µl fresh *Taq* DNA polymerase.

The quantity of DNA should be between 0.1 to 0.3 μg.

In subsequent iterations of this step, discrete products should be observed. Alternatively, the results of the agarose gel may suggest strategies for interventional troubleshooting (see Commentary). For example, a high background may indicate either primer hydrolysis or the need for increasing the stringency of the preceding hybridization step (i.e., decreasing tester relative to driver).

Change adapter on the difference product

47. Combine the contents of the two PCR tubes in one microcentrifuge tube (four tubes for the 96-well format). Extract and isopropanol precipitate as described in step 13.
48. Dissolve the pellet in 80 μl TE buffer. Determine DNA concentration by 2% agarose gel electrophoresis (*UNIT 2.5A*), and adjust to 0.1 μg/μl.
49. Digest 5 μg difference product (50 μl) with 10 U/μg chosen restriction enzyme (step 1) in a total volume of 200 μl. Bring volume of digested product up to 400 μl with water.
50. Extract and ethanol precipitate as described (step 2).
51. Resuspend DNA pellet at 0.1 μg/μl in TE buffer. Take 10 μl (1 μg) DNA solution and directly ligate to primer set E in a volume of 30 μl as described in steps 4 to 6.

    Changing primer sets between each round of RDA ensures that selective subtractive/kinetic enrichment of unique tester DNA restriction fragments will occur from newly ligated primer and not from uncleaved primer carried over from the previous rounds.

52. Dilute the ligated difference product to 1.25 ng/μl with TE buffer.

    For HindIII representation, dilute to 2.5 ng/μl with TE buffer.

    Always ligate 1 μg tester, then serially dilute the ligation product to a concentration such that 40 μl will give the appropriate amount of tester for the selected tester:driver hybridization ratio.

Perform subsequent subtractive/kinetic enrichment steps

53. For a second subtractive/kinetic enrichment, mix 40 μl (50 ng) adapter-ligated difference product (100 ng for *Hin*dIII representation) and 80 μl (40 μg) of driver amplicon DNA digest. Proceed through subtractive/kinetic enrichment exactly as outlined in steps 28 to 51 except substitute E for O primers/oligomers and dilute the ligated difference product to 2.5 pg/μl (10 pg/μl for *Hin*dIII representation).

    The second hybridization is done at a tester:driver ratio of 1:800 (1:400 for HindIII representations).

54. For a third subtractive/kinetic enrichment, mix 40 μl (100 pg) difference product from the second subtractive/kinetic enrichment (400 pg for *Hin*dIII representation) and 80 μl (40 μg) driver amplicon DNA digest. Proceed exactly as outlined in steps 28 to 51 using O primers/oligomers.

    The third hybridization is done at a tester:driver ratio of 1:400,000 (1:200,000 for HindIII representations).

55. *For HindIII*: Use 5 pg difference product from the third subtractive/kinetic enrichment (tester:driver ratio of 1:8,000,000). Again, proceed through steps 27 to 51 of the protocol, except substitute E for O primers/oligomers, and use 27 cycles in the final PCR of the selective amplification (step 44).

    For HindIII representation sometimes this fourth subtractive/kinetic enrichment is needed.

56. Clone products following gel isolation (*UNIT 2.6*) or use shotgun cloning and subsequent sequencing (*UNIT 7.1*).

cDNA REPRESENTATIONAL DIFFERENCE ANALYSIS

cDNA RDA works under the same principles as RDA of genomic DNA, and requires only minor modification from the procedure described above (see Basic Protocol 1). Two RDAs may be performed at the same time with the testers and drivers reversed in order to detect both induced as well as suppressed transcripts. There are two other modifications to Basic Protocol 1. The first is the substitution of *Dpn*II or its isoschizomer *Sau*3AI, as the restriction endonuclease. *Dpn*II is a four-base recognition enzyme that is compatible with the *Bgl*II and *Bam*HI primers listed in Table 25B.7.1. The second is the use of different ratios of tester to driver in the sequential hybridizations. For cDNA RDA, the ratios of 1:10, 1:100, 1:500, and 1:25000 may be used (see Table 25B.7.2 for ranges of tester:driver ratios; Pastorian et al., 2000).

Table 25B.7.2 Tester:Driver Hybridization Stringencies for cDNA RDA

Subtractive/kinetic enrichment	Range of tester:driver ratios
Round 1	1:10–1:50
Round 2	1:100–1:500
Round 3	1:1000–1:5,000
Round 4	1:10,000–1:50,000

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

EE × 3 hybridization buffer

30 mM 4-(2-hydroxethyl)-1-piperazinepropanesulfonic acid (EPPS), pH 8.0 at 20°C
3 mM EDTA
Store up to 6 months at room temperature.

RDA PCR buffer, 5×

335 mM Tris·Cl, pH 8.8 at 25°C (*APPENDIX 2*)
20 mM $MgCl_2$
80 mM $(NH_4)_2SO_4$
50 mM 2-mercaptoethanol
0.5 mg/ml BSA
Store up to 6 months at −20°C.

COMMENTARY

Background Information

Representational difference analysis (RDA) was first described in 1993 and has been used to detect polymorphisms between individuals, positional synteny between species, and genetic lesions in neoplasms (Lisitsyn et al., 1993; Lisitsyn and Wigler, 1995; Lowrey et al., 2000). In addition to finding genomic alterations, RDA has been successfully used to identify exogenous sequences from DNA-based infectious agents (Chang et al., 1994). While RDA was original applied to genomic DNA, the versatility of the technique allowed minor modifications in the protocol for the examination of differences in gene expression (Hubank and Schatz, 1994; Bakin and Curran, 1999; Reick et al., 2001; Shields et al., 2001) as well as the identification of new RNA viruses (Nishizawa et al., 1997; Birkenmeyer et al., 1998).

RDA has advantages and limitations when compared to other techniques used to detect

differences in genomic content. The first generation technique of subtractive hybridization requires large amounts of starting DNA and is inefficient, usually allowing only a 1:100-fold enrichment of target sequences. This is due to the complexity of eukaryotic genomes in which hybridization of complementary sequences cannot go to completion. Therefore, only very long or abundant sequences can be isolated. RDA circumvents this problem by the incorporation of a simplification step in which only a representation of the genome is used in the analysis. The simplification process is based on restriction endonuclease digestion of the genome and is accomplished by selective amplification of digested DNA fragments with lengths amenable to PCR followed by physical size selection. This simplification is key to successful RDA, but its disadvantage is that not all of the potential differences between two genomes will be found.

Many techniques are available for scanning differential gene expression, whether to ascertain changes that occur in development and differentiation, or that are associated with disease phenotypes. These include differential display, (*UNIT 25B.3*) cDNA array, serial analysis of gene expression (SAGE; *UNIT 25B.6*), and rapid analysis of gene expression (RAGE). In a novel combination of two techniques, RDA is performed first to generate products used as hybridization probes which are then applied to cDNA microarrays (Geng et al., 1998). Consideration must be given to the strengths and weakness of each tool in individual applications. The main advantages of using RDA are that the analysis is not limited to known sequences, it is efficient, and it is affordable for even small laboratories.

Critical Parameters and Troubleshooting

General considerations

DNA RDA is dependent on the generation of different DNA restriction fragments between driver and tester after restriction endonuclease digestion. Furthermore, the extra DNA fragment(s) must be found in the tester and not the driver, and must be within the size range for standard PCR amplification. Therefore, if the targeted genetic change does not result in a unique DNA fragment after digestion, then the change cannot be detected. In the case of DNA RDA, it is critical that the two samples to be compared are extracted from tissues or cells of nearly identical genetic background. To look for polymorphism, tissues from closely related individuals of the same gender may be used. To look for genetic changes associated with a neoplastic phenotype, tumor and normal tissue from the same individual is appropriately matched, unless the genetic change is germline. Although translocations may be identified whether the neoplastic tissue is used as the driver or tester, deletions require the neoplastic DNA to be used as driver. When the nature of the genetic change is not known, it is reasonable to perform two RDA with the samples switched from their designation as driver or tester.

Several issues arise when hunting for a microbial agent. The agent's genome must be large enough to offer a DNA fragment which when digested is big enough to PCR, and the genome must go through a DNA stage in its life cycle. RNA viruses must be pursued using cDNA RDA. Optimally, samples are acquired in a sterile manner and are free from contaminating organisms. In particular, epithelial or mucosal surfaces should be dissected off prior to DNA extraction. Diseases primarily involving such tissues are difficult to analyze by RDA unless existing microbial flora is matched. Lastly, the infected tissue should always be used as the tester, keeping in mind that the infection may be disseminated. In a related cautionary, when working with cell lines, ensure that no mycoplasma infection is present in cultures and that transformed cell lines are not generated by viral infections (i.e., herpesviruses, papillomaviruses, or adenoviruses).

The use of PCR in RDA necessitates implementation of procedures that guard against DNA contamination. If RDA is performed repetitively, all work areas and surfaces should be monitored regularly for occult adapter-ligated products. This can be done with swipe tests followed by PCR with the O and E 24-mers. PCR preparation, amplification, and analysis should be isolated from each other if possible, dedicated micropipettors should be used, and reagents should aliquoted and changed frequently.

Amplicon preparation

In both DNA and cDNA RDA, the quality of the starting material is important. Tissues or cells used to generate tester and driver DNA should be subjected to the same harvesting, storage, and DNA extraction conditions. Use methods for DNA preparation which give relatively pure DNA to ensure complete digestion.

The amount, the completion of digestion, as well as the integrity of DNA should be assessed by agarose gel electrophoresis to confirm that smears of tester and driver DNA in the initial steps prior to hybridization are comparable in both intensity and size distribution. Agarose gel electrophoresis is the preferred method for evaluating products in the protocol, since this method allows not only concentration determination, but also visualization of DNA integrity. When preparing cDNA, any standard protocol or kit may be used; however, be aware that some reverse transcriptases may contain minute amounts of contaminating vector which can give false positive results. To ensure the highest quality full-length cDNA, poly(A) RNA should be immediately subjected to reverse transcription and second-strand cDNA synthesis with no intermediate storage or precipitations.

If the amount of amplicon generated is suboptimal, several more cycles of PCR may be performed with the addition of new *Taq* DNA polymerase; however, PCR-introduced distortions of representations can be expected to be more pronounced at higher cycle numbers.

Subtractive/kinetic enrichment

In every round of subtractive hybridization, the amount of driver DNA remains constant. The amounts of tester (the product from the previous round) will diminish round by round to ultimately yield only the difference product or the differentially expressed targets. For DNA RDA, increasing stringency occurs with successive tester:driver ratios of 1:100, 1:800, 1:400,000, and 1:8,000,000. The tester:driver hybridization ratios may be modified, particularly when performing cDNA RDA to detect rare transcripts or smaller fold differences in expression between tester and driver. If no DNA products appear as bands by agarose gel electrophoresis in the later rounds of RDA, it may help to start either the particular hybridization round or the entire RDA again with a less stringent tester:driver hybridization ratio (relatively more tester DNA). If too much background smearing occurs in later rounds of RDA and primer problems have been ruled out (see below), then a more stringent tester:driver hybridization ratio in the preceding round may help.

No difference products

RDA requires the generation of restriction fragments between 200 to 1000 bp to ensure optimal PCR amplification. Because of this simplification step, a particular restriction strategy may fail to find sought after differences. Therefore, if no difference products are isolated after iterative rounds of kinetic/subtractive enrichment, alternate restriction endonucleases may be tried. The placement in the tester sample of an internal control with known restriction characteristics at the beginning of an RDA experiment can be used; however, to prevent preferential amplification of the internal control, the internal standard should be spiked at sufficiently low concentrations (<1:100,000 on a weight basis).

Too many difference products or high background

HPLC purification of oligomers is critical for minimizing false positive RDA bands (O'Neill and Sinclair, 1997). Additionally, repeated thawing and freezing of primers in aqueous solution results in increased primer hydrolysis and contributes to mispriming during PCR amplification. This may result in increased false positives, increased rounds required to isolate true difference products, and excessive background smearing. Primers can be stored in lyophilized aliquots to circumvent this problem.

Anticipated Results

The RDA protocol selectively enriches for unique DNA sequences in the tester DNA sample. Upon completion of RDA, enriched populations of DNA can be visualized on agarose gel electrophoresis as a few or several distinct bands that range usually from between 150 to 800 bp. Although a significant background smear with only poorly identifiable bands may be seen at the end of the first round of kinetic/subtractive enrichment, successive rounds of enrichment should result in sharper bands with clean backgrounds. Even if discrete bands appear in the first or second rounds of RDA, three or more rounds are typically required to minimize background amplimers or stochastically amplified false positives. Authentic bands can then be cloned by a variety of different approaches. Sequence analysis of clones should reveal authentic endonuclease restriction sites at the termini of the inserts. It is not unusual to identify more than one discrete DNA fragment from each band; however, the majority of the clones should contain a single true positive difference product.

Time Considerations

Preparation of amplicons and representations requires 4 days. Each round of DNA

kinetic/subtractive enrichment also requires 4 days. If three rounds are performed, an RDA experiment exclusive of DNA and cDNA preparation or subsequent cloning can be completed in 16 days. Four rounds require 20 days. With some consideration for life's distractions, an RDA experiment can be performed in 4 weeks.

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Gene Expression Analysis of a Single or Few Cells

The need to analyze rare or even single cells is based on the dynamic nature of tissue differentiation and regeneration, the initiation and propagation of disease processes in multicellular organisms, and the functional diversity of individual cells. Gene transcription is the most important regulatory mechanism by which a phenotype and functional state of a cell is determined. Therefore, qualitative and quantitative assessment of mRNA abundance is not only a first step into the nature of biological processes but is easier to investigate in a comprehensive way than protein expression when small cell numbers are used.

In this unit, a protocol that allows a semi-quantitative analysis of gene expression of a single cell and a quantitative representation of expressed genes from >10 to 30 cells is described. This unit concentrates on the amplification procedure (see Basic Protocol 1) and less on the cDNA array hybridization. However, a basic protocol (see Basic Protocol 2) for array hybridization on nylon filters is provided because such filters are available in every laboratory without the need of additional expensive equipment. As tissue samples contain many different cell types in variable amounts, their analysis often requires microdissection of the tissue to isolate the specific cell types. Therefore, additional information on how to isolate mRNA from very small tissue samples such as biopsies and laser-microdissected material from cryosections (see Alternate Protocols 1 and 2) is given. Finally, a simple procedure to prepare the data for statistical analysis is also provided (see Basic Protocol 3).

STRATEGIC PLANNING

This unit deals with the handling of minute amounts of mRNA. Therefore, two "natural foes," contamination and RNA degrading enzymes (RNases; see *UNIT 4.1* for additional details), will be encountered. Contamination can be reduced by working under a laminar-flow clean bench that has never been exposed to PCR-amplified DNA or cloned DNA, and that is preferably located in a room apart from laboratories where DNA is handled. It is recommended to always use filter tips for solutions and to take care not to contaminate pipets or other devices with DNA from other rooms. Unfortunately, contamination might still occur since many enzymes (in particular, reverse transcriptase) contain traces of bacterial DNA/RNA that will be co-amplified with the desired single-cell mRNA. For many assays, this bacterial DNA will not interfere, but may be a potential source of trouble. Degradation of RNA by RNases can be avoided by the use of powder-free gloves (changing them frequently) and being cautious when preparing buffers. RNase inhibitors are not added because they are frequently derived from human placenta and might therefore be contaminated with human nucleic acids. Working quickly and placing probes on ice is also recommended.

GLOBAL AMPLIFICATION OF SINGLE-CELL cDNA

BASIC PROTOCOL 1

This PCR-based protocol has been developed for maximal sensitivity of transcript detection. This raises the concern of exponential-error transmission, which will be discussed in detail along with the means that have been undertaken to reduce this error. However, one has to be aware that by using this method an exact quantification of the transcripts from a single cell is not possible; rather, semi-quantitative results are obtained.

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To achieve maximal sensitivity, conditions were sought to avoid unnecessary loss of mRNA during the precipitation steps. Enzymatic activity of the reverse transcriptase or *Taq* polymerase should not be compromised by using less than an optimal supply of substrates or by inadequate buffers.

The basic goal of this protocol is to introduce two binding sites for PCR primers into cDNAs representing transcripts, allowing amplification of each transcript uniformly (Fig. 25B.8.1). The first primer-binding site is contained within a flanking region that lies at the 5′-end of a random cDNA synthesis primer or an oligo dT primer. The second is introduced through a tailing step using terminal deoxynucleotide transferase (TdT). Therefore, three enzymatic steps are required—cDNA synthesis, tailing, and PCR. The use of a random primer has two advantages. First, it enables amplification of 5′ regions that might be of interest (e.g., when mutations are studied), and second, it leads to production of cDNAs of lengths that are optimal for PCR amplification. However, for cDNA synthesis with a random primer, it is important to remove most of the rRNA and tRNA, which comprise >95% of total cellular RNA. Therefore, mRNA is purified using

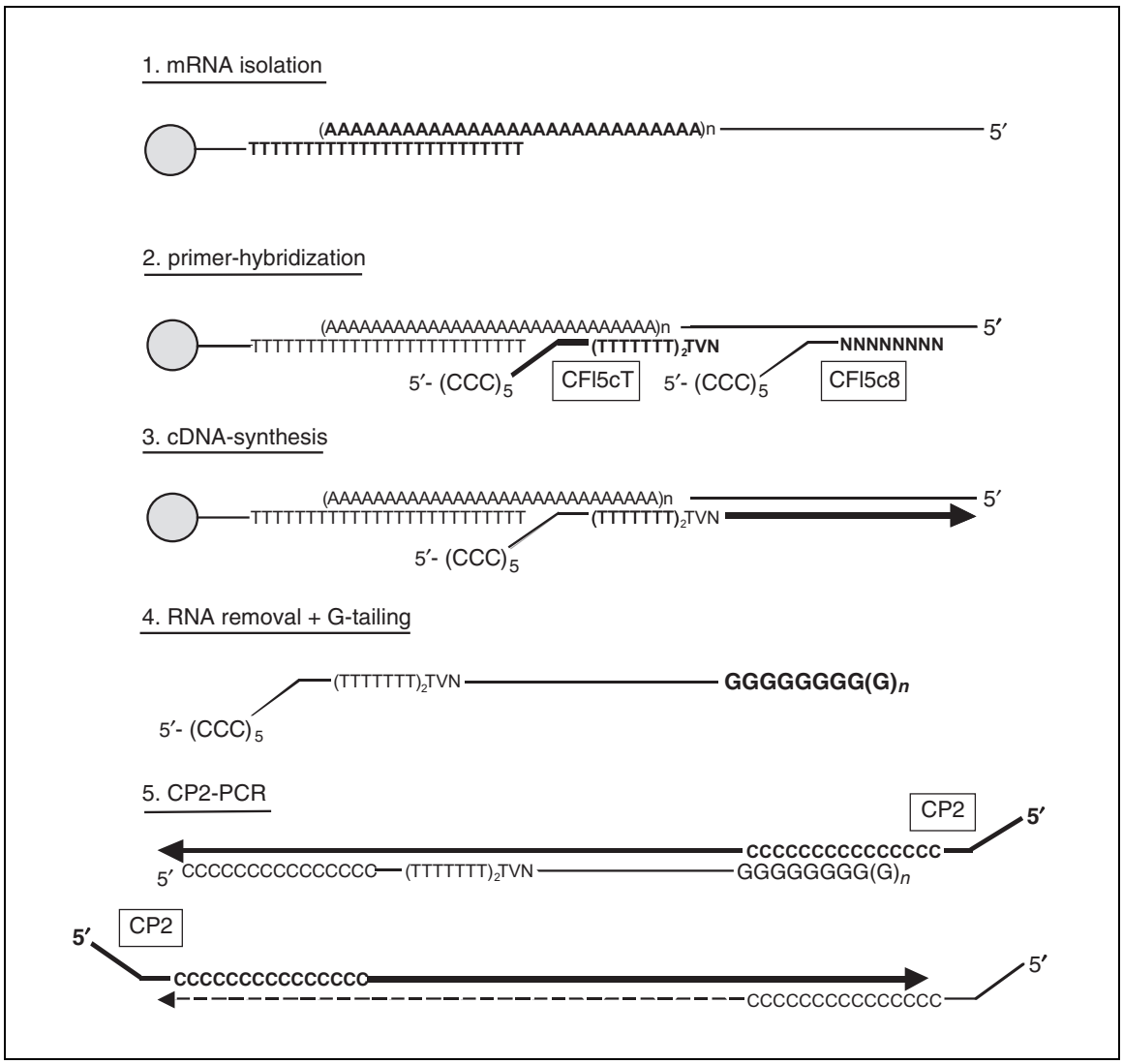

Figure 25B.8.1 Global amplification of mRNA from a few or single cells. mRNA is captured by paramagnetic beads (**1**), and primed using random and oligo dT primers containing a poly C flanking region (**2**). cDNA synthesis starts from both primers (**3**; CFL5c8 is omitted in **3** and **4**). After RNA removal, a poly G tail is added by TdT. Using the poly C containing CP2 primer, all sequences can be amplified (**5**).

paramagnetic oligo-dT beads. While the mRNA is bound to the beads, reaction buffers can easily be changed without loss of mRNA or cDNA. This allows using optimal (i.e., high) concentrations of cDNA primers and nucleotides during cDNA synthesis without interference with the subsequent tailing reaction. To avoid loss of transcripts, do not contaminate the reaction with RNases because the mRNA holds the newly synthesized cDNA to the bead. After cDNA synthesis and before starting the tailing reaction, the unbound cDNA synthesis primers and unincorporated dNTPs have to be washed out. Tailing is performed in a KH_2PO_4 buffer that, unlike the provided potassium-cacodylate buffers, does not inhibit the subsequent PCR reaction, which is set up in the same reaction tube without discarding the tailing buffer.

Random primers were originally used because they reduced the length of an amplicon and allowed amplification of 5′-sequences. These random primers, combined with oligo-dT primers, slightly improve the results when single cells are used (CFL5 primer mix). However, when higher cell numbers (>100) are used, it appears that random primers alone work at least as well as the combination. For single cells, a random octamer increases the average fragment length, compared to a random hexamer, by ~100 to 200 bp. Due to the increasing number of commercially available oligo arrays that are restricted to the 3′-end, it might be advantageous to use oligo dT primers alone. The authors' first experiments indicate that the $CFl5CT_{(24)}$ primer should be used in this instance.

Materials

Oligo dT kit (Dynal) including:
  Dynabeads Oligo $(dT)_{25}$
  Washing buffer containing LiDS
  Lysis buffer
Phosphate-buffered saline (PBS; *APPENDIX 2*)
5× RT buffer (Life Technologies)
0.1 M DTT (Life Technologies)
10% (v/v) Igepal
cDNA synthesis primers:
  For mRNA amplification for ≥100 cells:
  CFL5C6: 5′-$(CCC)_5$ GTC TAG ANN NNN N-3′ (200 μM)
  For single cells and 5′ and 3′ coverage:
  CFl5C8: 5′-$(CCC)_5$ GTC TAG ANN NNN NNN-3′ (200 μM)
  CFl5CT: 5′-$(CCC)_5$ GTC TAG ATT TTT TTT TTT TTT TVN-3′ (100 μM)
  CFL5 primer mix: 1 vol CFl5c8 (200 μM) + 1 vol CFl5cT (100 μM)
  For the use of 3′-restricted oligo arrays:
  $CFl5CT_{(24)}$: 5′-$(CCC)_5$ GTC TAG ATT $(T)_{22}$VN-3′
10 mM and 200 μM dNTPs
Reverse transcriptase (Superscript II; Life Technologies)
Igepal wash buffer (see recipe)
Tween 20 wash buffer (see recipe)
40 mM $MgCl_2$
2 mM dGTP
200 mM KH_2PO_4
Tailing wash buffer (see recipe)
Mineral oil
Terminal deoxynucleotide transferase (TdT; Amersham Pharmacia Biotech)
Expand Long Template (ELT) PCR system (Roche Diagnostics) including:
  10× ELT buffer 1 (17.5 mM $MgCl_2$)
  3.5 U/μl DNA polymerase mix

20% (v/v) formamide
PCR primer, CP2: 5′- TCA-GAA-TTC-ATG-CCC-CCC-CCC-CCC-CCC-3′ (24 μM)
1× PCR buffer (Sigma)
Primers for β-actin: 5′- CTA CGT CGC CCT GGA CTT CGA GC-3′ and 5′-GAT GGA GCC GCC GAT CCA CAC GG-3′
Primers for EF-1α: 5′- GCA GTG CAC ACA CAG AGG TGT A-3′ and 5′- CTA CCG CTA GGA GGC TGA GCA A-3′
0.75 U *Taq* DNA polymerase (Sigma)

Magnet separation apparatus for 0.2-ml tubes (Dynal)
0.2-ml PCR tubes
15- to 50-ml tubes
Roller-bottle apparatus or other rotisserie-type rotator
Thermal cycler
Hybridization oven or other rotator with temperature control

Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5A*)

Lyse cells and isolate mRNA

1. Wash beads two times in an equal volume of washing buffer containing LiDS using the magnet.

   Dynal beads are supplied as a solution and have to be washed using the magnet prior to use. Resuspend beads in adequate volume of lysis buffer to which the cells or tissue biopsies are added.

   The beads must completely adhere to the side of the tube at the site of the magnet before the supernatant is removed to avoid loss of beads. This wash procedure can take several minutes. Do not forget to prepare beads for the negative control.

2. Resuspend beads in an equal volume of lysis buffer. The amounts of lysis buffer and beads depend on the cell number. Table 25B.8.1 suggests the volumes of lysis buffer and beads to use for specific numbers of cells.

3. Pick cells in 1× PBS (*APPENDIX 2*) in the smallest possible volume. Pick single cells in a 1- to 2-μl volume and add to the beads in lysis buffer in a 0.2-ml PCR tube.

   Individual cells can be isolated from suspensions using a 2-μl automatic pipettor and an inverted microscope.

   Cell numbers >3000 in one reaction tube should be avoided because the released genomic DNA will clump the beads and prevent successful isolation of mRNA. When more cells are used, either use up to 500 μl of lysis buffer with 50 μl of beads, use aliquots, or isolate total RNA first by classical protocols (e.g., UNIT 4.1) and add the RNA (1 to 10 μg total RNA) to the beads.

4. Place the 0.2-ml PCR tubes in a 15- to 50-ml tube and rotate the lysate for 30 min at 4° to 20°C (room temperature) in a roller-bottle apparatus.

   Rotation ensures that the beads remain suspended.

   If desired, freeze the sample after this step at –80°C. The authors have stored samples for up to 12 months without any negative effect. On continuation, resuspend the beads after thawing and rotate for 5 min.

Table 25B.8.1 Volumes of Beads and Lysis Buffer for Given Numbers of Cells

No. of cells	Oligo dT beads	Lysis buffer
1–10	10 μl	10 μl
11–50	30 μl	30 μl
51–300	50 μl	50 μl
>300–3000	50 μl	50–200 μl

Table 25B.8.2 cDNA Synthesis Mixes

No. of samples	1	2	3	4	5	6	7	8	9	10
cDNA synthesis mix I[a]										
5× first strand buffer	2	4	6	8	10	12	14	16	18	20
0.1 M DTT	1	2	3	4	5	6	7	8	9	10
10% Igepal	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
H_2O	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
cDNA synthesis primers	6	12	18	24	30	36	42	48	54	60
cDNA synthesis mix II[a]										
5× first strand buffer	2	4	6	8	10	12	14	16	18	20
0.1 M DTT	1	2	3	4	5	6	7	8	9	10
10 mM dNTP	1	2	3	4	5	6	7	8	9	10
H_2O	5	10	15	20	25	30	35	40	45	50
Reverse transcriptase	1	2	3	4	5	6	7	8	9	10

[a]All solution volumes are indicated in microliters.

Synthesize cDNA

5. Prepare cDNA synthesis mix I and II (see Table 25B.8.2) on ice while the beads are rotating. Add the reverse transcriptase to mix II just before use.

   Never use a reverse transcriptase with RNase H activity.

6. Add an equal volume of Igepal wash buffer to the cell lysate containing the mRNA bound to the beads and place tube in the magnet. Remove supernatant after the beads have completely adhered to the tube at the site of the magnet. Resuspend beads carefully in 20 µl Tween 20 wash buffer. Transfer to a fresh 0.2-ml tube, place in the magnet, and remove the supernatant after complete adhesion of beads to the magnet.

   The multiple washing steps as well as the change of the reaction tube serve to remove the LiDS-containing buffer, since even small traces of LiDS can inhibit reverse transcription. It is very important to allow complete adhesion of the magnetic beads to the tube wall at the site of the magnet to avoid loss of cDNA. Note that collection of the supernatant and storage at −20°C may be desired because it contains the genomic DNA that can be used for additional analyses at a later time.

7. Resuspend beads in cDNA synthesis mix I and allow primers to anneal for 2 min on the bench at room temperature, then add mix II (remember to add the RT in mix II). Immediately start cDNA synthesis by placing the tubes in a hybridization oven for 45 to 60 min at 44°C with rotation.

   It is important to rotate so that the beads remain suspended.

   The authors tape the 0.2-ml sample tubes to pre-heated hybridization bottles.

8. Prepare tailing mix (see Table 25B.8.3).

9. Place tubes in the magnet and remove supernatant. Wash beads one time in 20 µl tailing wash buffer. Pre-heat thermal cycler to 94°C.

   After cDNA synthesis and before starting the tailing reaction, the unbound cDNA synthesis primers and unincorporated dNTPs have to be washed off. Therefore, meticulously remove all of the cDNA synthesis solutions by carefully pipetting, because dNTPs and primers will interfere with the tailing reaction.

Table 25B.8.3 Tailing Mix[a]

No. of samples	1	2	3	4	5	6	7	8	9	10
40 mM $MgCl_2$	1	2	3	4	5	6	7	8	9	10
1 mM DTT	1	2	3	4	5	6	7	8	9	10
2 mM dGTP	1	2	3	4	5	6	7	8	9	10
200 mM KH_2PO_4	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
H_2O	6.5	13	19.5	26	32.5	39	45.5	52	58.5	65

[a]All solution volumes are indicated in microliters.

Table 25B.8.4 PCR Mixes for Global Amplification[a]

No. of samples	1	2	3	4	5	6	7	8	9	10
PCR-mix I										
Roche buffer 1	4	8	12	16	20	24	28	32	36	40
20% formamide	7.5	15	22.5	30	37.5	45	52.5	60	67.5	75
H_2O	24	48	72	96	120	144	168	192	216	240
PCR-mix II										
24 µM CP2 primer	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25
10 mM dNTP	1.75	3.5	5.25	7	8.75	10.5	12.25	14	15.75	17.5
Taq long template	1.5	3	4.5	6	7.5	9	10.5	12	13.5	15

[a]All solution volumes are indicated in microliters.

Tail cDNA

10. Resuspend beads in tailing mix and add 40 µl mineral oil on the surface. Place the 0.2-ml tubes in the preheated thermal cycler and denature RNA-DNA hybrids for 5 min at 94°C. Immediately chill on ice.

    This step serves to generate single-stranded cDNA, which is tailed with high efficiency in contrast to RNA-DNA hybrids. (After denaturation, the cDNA is no longer bound to the magnetic beads but is now found in the supernatant.) The following tailing and PCR procedure will take place with the beads in the tube.

11. Add 10 to 15 U TdT, mix thoroughly, and start tailing in a thermal cycler programmed for 60 min at 37°C, then 22°C indefinitely.

    Tailing is complete after 1 hr, but can be extended overnight at 22°C, whenever necessary.

12. Inactivate TdT by incubating cDNA at 70°C for 5 min.

Amplify by PCR

13. Prepare PCR mix I and II on ice (see Table 25B.8.4).

14. After inactivation of TdT, add PCR mix I to the aqueous phase under the mineral oil. Incubate for 30 sec at 78°C.

15. Add 5.5 µl mix II, then carry out the amplifications in a thermal cycler with the following parameters:

1 cycle:	30 sec	78°C
19 cycles:	15 sec	94°C
	30 sec	65°C
	2 min	68°C

continued

20 cycles:	15 sec	94°C
	30 sec	65°C
	2.5 min + 10 sec/cycle	68°C
1 cycle:	7 min	68°C
	indefinitely	4°C.

The separation of mix I and II serves for the hot-start procedure. Add the largest solution volume first, which consists of buffer and water. After 78°C has been reached, add the primers, nucleotides, and enzymes. Taq long template is one of several available mixtures of a highly processive DNA polymerase (Taq-polymerase) and a proof-reading enzyme with 3′-5′ exonuclease activity (Pwo-polymerase). The exonuclease activity would degrade the single-stranded CP2 primer in absence of dNTP, which consequently has to be included in mix II. The reason for the hot start is to avoid unspecific priming and extension of the CP2 primers (that bound to the single-stranded cDNA at low temperatures) until 94°C is reached. The longer extension time in cycles 20 to 39 is due to the increased amount of product.

16. Store sample at –20°C.

Evaluate global amplification and validate genes

17. Check 3 to 5 µl of the primary PCR on a 1.5 % agarose gel for the presence of a smear in the range of 300 to 2000 bp.

18. Test amplification success by performing gene-specific PCR on at least two housekeeping genes.

    For human cells, use the primers for β-actin and EF-1α (see Materials) in the conditions outlined below (see step 19). For other species, choose/design primers specific to housekeeping genes of those species.

    To test amplification success, perform gene-specific PCRs for selected genes. Each gene-specific PCR should be individually optimized. For most transcripts, best results will be obtained after dilution of the primary amplifications in water (1:10). As the length of the amplified cDNA is usually <1000 bp, choosing primers that amplify sequences of 150 to 200 bp is recommended, as this size range produces the best results.

19. Make up a PCR reaction containing 2.5 ng of each cDNA in a 25-µl reaction containing 1× PCR buffer (Sigma), 200 µM dNTPs, 0.4 µM of each primer (β-actin or EF-1α), and 0.75 U *Taq* polymerase. Carry out the amplifications in a thermal cycler with the following parameters:

1 cycle:	2 min	94°C
	30 sec	58°C
	2 min	72°C
14 cycles:	40 sec	94°C
	30 sec	58°C
	20 sec	72°C
15-45 cycles:	40 sec	94°C
	30 sec	58°C
	30 sec	72°C
1 cycle:	40 sec	94°C
	30 sec	58°C
	2 min	72°C
	indefinitely	4°C.

The number of cycles in the main part of the amplification can be 15 to 45, depending on the transcript abundance.

20. Run PCR products on a 2% agarose gel containing 0.5 µg/ml ethidium bromide.

ALTERNATE PROTOCOL 1

EXTRACTION OF mRNA FROM SMALL TISSUE BIOPSIES

This protocol is used to isolate and amplify mRNA from small biopsies that are obtained during diagnostic clinical procedures and do not undergo laser microdissection (*UNIT 25A.1*). The fresh biopsy is immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen or at −80°C until lysis and mRNA preparation is performed.

Additional Materials *(also see Basic Protocol 1)*

Biopsy sample
Liquid nitrogen
Dry ice
Mortar and pestle

1. Use only a small piece of the biopsy sample with a size of 1 to 1.5 mm in diameter.
2. Using a mortar and pestle, crush the frozen tissue sample in liquid nitrogen.

   Prior to using the mortar and pestle, destroy all nucleic acids by UV irradiation. Expose the internal surface of the mortar to UV light in a transilluminator or hold it close to a UV light source (254-nm wavelength) for 10 to 15 min. For the pestle, in order to expose the whole surface, it will be necessary to turn it, as only DNA lying in the direct path of the light will be destroyed by the UV irradiation (also see APPENDIX 3F for sterile technique).

   Thawing of the sample must be avoided under all circumstances! Therefore, place the mortar on dry ice and frequently pour liquid nitrogen over the sample.
3. Add the powdered sample directly to 50 µl of prepared Dynal beads (see Basic Protocol 1, steps 1 and 2) and rotate lysate as in Basic Protocol 1, step 4.
4. Proceed with global amplification in Basic Protocol 1, steps 5 through 20.

ALTERNATE PROTOCOL 2

EXTRACTION OF mRNA FROM MICRODISSECTED SAMPLES

Laser microdissection is the cleanest way to isolate selected morphologically defined cell groups from tissue sections. However, it is also possible to scratch the tissue area with a glass needle of which the tip is then broken into the lysis buffer. The authors use the PALM Laser-MicroBeam System (PALM) that first cuts the selected area by a laser beam and then catapults it into the lid of the reaction tube (see Fig. 25B.8.2). Other laser microdissection systems (see *UNIT 25A.1*) should work equally as well, as long as the isolation does not change the composition of the lysis buffer. The combination with Basic Protocol 1 (Global Amplification) and Basic Protocol 2 (Non-Radioactive Gene Expression Analysis on Nylon Arrays) enables quick analysis of global gene expression from 30 to 200 cells from 5-µm sections.

Materials *(also see Basic Protocol 1)*

Resectioned tissue snap-frozen in liquid nitrogen and stored at −80°C (see Alternate Protocol 1)
OCT embedding compound (Tissue-Tek, Miles; also see *UNIT 25A.1*)
Mayer's hematoxylin solution (Sigma)
70%, 95%, and 100% ethanol
Lysis buffer from Oligo dT kit (see Basic Protocol 1)

Cryostat
Slides for the PALM Laser-MicroBeam System (PALM)
PALM Laser-MicroBeam System (PALM)

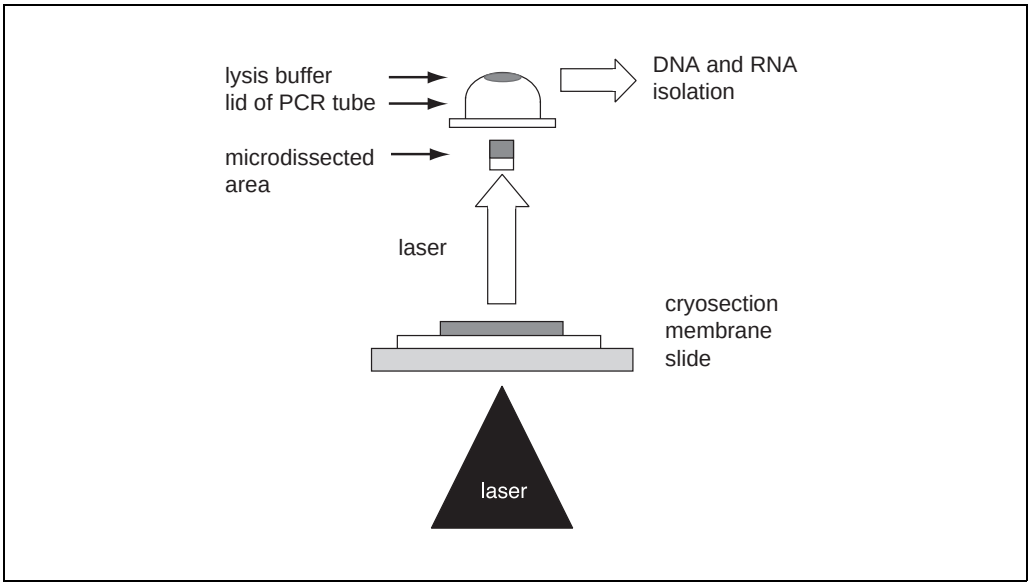

Figure 25B.8.2 Isolation of small tissue samples by laser microdissection and catapulting using the PALM system.

1. Embed the tumor sample in OCT embedding medium (*UNIT 25A.1*) and cut the sample to 5-µm thick slices on slides for the PALM Laser-MicroBeam System using a cryostat.
2. Place the slides in Mayer's hematoxylin solution for 45 sec, in water for 5 min, and in distilled water for 1 min.
3. Dehydrate sections in 70%, 95%, and 100% ethanol for 60 sec in each concentration.
4. Dry stained tissue sections overnight at room temperature.

   The slides are ready for the Laser-MicroBeam System.

   For the PALM Laser-MicroBeam System, the sections have to be completely dried, otherwise the heat generated by the laser beam will be transmitted, boil the tissue, and destroy the mRNA. If using a different microdissection system, individually establish the conditions and parameters.
5. To catch the catapulted tissue area in the lid of a PCR reaction tube, pipet 5 µl lysis buffer on the inner wall of the lid.
6. Centrifuge the lysed tissue (mRNA and DNA) at maximum speed and proceed with mRNA isolation and global amplification (see Basic Protocol 1).

NON-RADIOACTIVE GENE EXPRESSION ANALYSIS ON NYLON ARRAYS

BASIC PROTOCOL 2

This protocol allows one to assay the expression of many genes whose mRNAs are represented in the amplification in Basic Protocol 1 without expensive equipment. It also assesses the complexity of sequences within the amplification, which can be helpful before proceeding to more detailed analyses. Test filters may be self-prepared by spotting 5 to 50 ng of each cDNA sequence (each should have a length of 300 to 700 bp) in 1 to 2 µl of 0.1 M NaOH on a positively charged nylon membrane. There are also several commercially available products. See Chapter 22 for methods to prepare and assay arrays on glass slides.

Materials

Expand Long Template (ELT) PCR system (Roche Diagnostics) including:
  10× ELT buffer 1 (17.5 mM $MgCl_2$)
  3.5 U/µl DNA polymerase mix
1/7 dNTP mix (see recipe)
20% formamide
CP2 primer: 5′- TCA-GAA-TTC-ATG-CCC-CCC-CCC-CCC-CCC-3′ (24 µM)
Digoxigenin-11-dUTP (Dig-UTP), alkali labile (Roche Diagnostics)
Sample
DIG Easy Hyb solution (Roche Diagnostics)
E.coli DNA
DNase I
Labeled probe
Herring sperm DNA (Invitrogen)
20× SSC
10% SDS
Development buffer 1 (see recipe)
Development buffer 2 (see recipe)
DIG Luminescent Detection Kit (Roche Diagnostics) containing:
  Blocking reagent
  750 U/ml anti-digoxigenin-AP (Fab fragment) antibody
  11.6 mg/ml CSPD
Tween 20 (Sigma)
Development buffer 3 (see recipe)

Thermal cycler
Nylon membrane containing an array of cDNAs (either self-prepared or commercially available)
Hybridization tubes
Hybridization oven or other rotator with temperature control
1.5-ml microcentrifuge tubes
Acetate sheets
Whatman 3MM filter paper
Biomax ML film (Kodak)

Label amplifications with Dig-UTP

1. Prepare PCR master mix as in Table 25B.8.5. Pipet 49-µl aliquots in sterile PCR tubes, add 1 µl from the sample (i.e., from the PCR product obtained in Basic Protocol 1, step 15) and program the thermal cycler with the following parameters:

1 cycle:	2 min	94°C
	4 min	68°C
10 cycles:	15 sec	94°C
	4 min	68°C
2 cycles:	15 sec	94°C
	4 min + 10 sec/cycle	68°C
1 cycle:	7 min	68°C

2. Determine the concentration of the amplified DNA (see *UNIT 2.6*, Support Protocol).

3. Prehybridize nylon array by placing the nylon membrane containing the cDNA array in a small hybridization tube, add 6 ml DIG Easy Hyb solution supplemented with 100 µg/ml *E. coli* DNA that has been digested with DNase I to a size of 100 to 1000 bp, and prehybridize for at least 6 hr at 45°C.

Table 25B.8.5 PCR Master Mix for Non-Radioactive Gene Expression Analysis[a]

No. of samples	1	2	3	4	5	6	7	8	9	10
10× ELT buffer 1	5	10	15	20	25	30	35	40	45	50
dNTP mix	1.75	3.5	5.25	7	8.75	10.5	12.25	14	15.75	17.5
20% formamide	7.5	15	22.5	30	37.5	45	52.5	60	67.5	75
24 µM CP2 primer	5	10	15	20	25	30	35	40	45	50
Dig-UTP	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25
H_2O	28	56	84	112	140	168	196	224	252	280
3.5 U/µl DNA polymerase mix	0.5	1	1.5	2	2.5	3	3.5	4	4.5	50

[a]All solution volumes are indicated in microliters.

Be aware that several commercial membranes are heavily contaminated with bacterial and/or plasmid DNA. The additional DNA in the hybridization solution serves not only to block all non-specific binding of labeled probe but also any amplified bacterial/plasmid DNA contaminating the enzyme preparations used to generate probes. All enzyme preparations contain traces of bacterial RNA/DNA that will be amplified by the highly sensitive amplification protocol and sometimes hybridize to bacterial/plasmid DNA on the filters. Therefore, even with the additional DNA, some poor arrays might not be usable. Always test the quality of the arrays by labeling and hybridizing a probe that has been amplified by the protocol in the absence of cellular RNA (negative control), in which contaminating DNA from the enzymes can be expected to be present as in the cell samples.

Note that although some favor the opposite nomenclature for array hybridizations, the authors use the term "probe" to refer to the labeled DNA in solution.

Add probe to membrane

4. Mix in a 1.5-ml microcentrifuge tube, 1 ml DIG Easy Hyb solution, 6 µg of the labeled probe from step 1, and 100 µg of herring sperm DNA. Denature 5 min at 94°C and immediately add to the prehybridization solution in the hybridization tube. Incubate with slow rotation at least 36 hr at 45°C.

It is important that the nylon membrane be completely covered with the hybridization solution before rotating. Otherwise, high non-specific backgrounds will result due to the drying of the membrane during hybridization. Therefore, adjust the amount of hybridization solution to add to the prehybridization accordingly.

Additionally, do not pour the concentrated probe directly onto the filter. This will result in high background.

Wash the membrane

5. Remove the hybridization solution and wash the membrane in the bottle and in the hybridization oven at 68°C using the following regimen:

1 min in 2× SSC + 0.1% SDS
1 min in 1× SSC + 0.1% SDS
15 min in 0.5× SSC + 0.1% SDS
30 min in 0.1× SSC + 0.1% SDS (two times)

Warm all solutions to 68°C prior to use in a water bath.

The hybridization mix can be stored at −20°C and re-used for additional filters. Before re-using the hybridization mix, denature the solution for 10 min at 80°C.

6. Wash the membrane in development buffer 1 for a few seconds at room temperature, then block in 25 ml development buffer 2 for 30 min with gentle agitation.

7. Dilute 2.5 µl anti-digoxigenin-AP (Fab fragment) antibody directly into the 25 ml development buffer 2 and incubate for an additional 30 min at room temperature.

8. Pour off development buffer 2 and wash two times, 15 min each, in development buffer 1 containing 0.3% Tween 20 at room temperature.

   This step will remove the unbound antibody.

Detect binding with chemiluminescent substrate

9. Prepare 50 ml of development buffer 3 and prepare 1 ml of chemiluminescent substrate by mixing 10 µl CSPD with 990 µl development buffer 3. Equilibrate the membrane for a few seconds in the remaining development buffer 3.

10. Place the membrane between two acetate sheets. Lift the top sheet of plastic and add 1 ml of the chemiluminescent substrate (from step 9), scattering the drops over the surface of the membrane. Carefully lower the top sheet of plastic without producing any bubbles.

11. Incubate on the bench 5 min at room temperature. Remove the membrane from the plastic sheets and place on a sheet of Whatman 3MM paper for a few seconds to remove excessive chemiluminescent substrate, then put the membrane back between two clean, dry acetate sheets.

    It is important to remove any excess moisture from the membrane. This avoids the development of background during film exposures up to 60 min. However, the membrane should not completely dry out because this would exclude any further use.

12. Incubate 15 min at 37°C and place the membrane on film to be exposed.

    The 37°C-incubation allows the alkaline phosphatase reaction to reach a steady state quickly. The authors recommend 15 min for the first exposure, then adjust the time according to the signal strength.

BASIC PROTOCOL 3

DATA ANALYSIS OF HYBRIDIZED cDNA ARRAYS

There are several ways to analyze and normalize the data obtained by gene-expression profiling with cDNA arrays. This protocol describes a method to measure differences of signal intensities of differentially expressed genes and to normalize the signal intensities to several housekeeping genes. See *UNIT 22.3* for other information regarding data analysis.

Materials

Photographic step tablet (Kodak)
Transparency scanner that can be calibrated (e.g., SNAPSCAN, Agfa)
Labscan software or equivalent (Scanwise v. 1.2.1, Agfa)
Array Vision software or equivalent (Clontech)
Excel software or equivalent (Microsoft)
SPSS software or equivalent (SPSS)

Perform intensity calibration of the scanner

1. Define the known density values from the photographic step tablet.

   To analyze the signal intensity on the X-ray film, it is important to measure its optical density. Signal intensity is usually measured in units, which do not necessarily represent the same "real-world" values in different images. It is important to calibrate a scanner before measuring the optical density of the signals. Therefore, by indicating raw intensity values in an image and defining their corresponding optical density, the system can be provided with the information it needs to convert its measurements to real-world quantities.

2. Scan the photographic step tablet in the grayscale mode values that have been entered.

   The Kodak No. 2 Photographic Step Tablet Standard values are provided as optical density values, starting at 0.05 and proceeding at 0.15-OD increments to 3.05 OD units. At least four calibration points are necessary to compute a calibration curve.

3. Compute a calibration curve.

   At least three different curve models are available. The linear option calculates the curve with the formula: $y = ax + b$; the quadratic option with the formula: $y = ax^2 + b$; and the log linear option with the formula: $y = a \log ((255 – x)/255) + b$. The authors recommend the log linear option.

Scan the developed films

4. Scan the grayscale of the developed films in the transmission mode with a resolution of at least 600 dpi.

5. Save files as MD GEL (*.gel), MCID (*.im), BRS (*.img), TIFF (*.tif), TIFF5 (*.tif), Fujix Bas Series (*.inf), Bio-Rad PA (*.img), Packard (*.tif), or MD Dataset (*.ds).

   Other data formats cannot be imported by the array vision software.

Analyze with software

6. Define a template according to the grid of the cDNA arrays that were used.

7. Import the scanned films as a data file into arrays vision.

8. Align the grid to the corresponding spots on the cDNA array.

9. Normalize the signals to the housekeeping genes present on the cDNA array.

   The average of the signals of the housekeeping genes is set to a value of one and the background to a value of zero.

10. Sample the template.

11. Export the gained data to MS Excel and/or SPSS for further statistical analysis.

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

Development buffer 1

100 mM maleic acid
150 mM NaCl, pH 7.5
Autoclave and store up to 6 months at room temperature

Development buffer 2

100 mM maleic acid
150 mM NaCl, pH 7.5
1% blocking reagent (DIG Luminescent Detection Kit, Roche Diagnostics)
Store up to 12 months at –20°C

Development buffer 3

100 mM Tris·Cl, pH 9.5 (*APPENDIX 2*)
100 mM NaCl
Prepare fresh just prior to use.

dNTP mix, 1/7

10 mM dCTP
10 mM dGTP
10 mM dATP
8.4 mM dTTP
Store up to 12 months at –20°C

Igepal wash buffer

50 mM Tris·Cl, pH 8 (*APPENDIX 2*)
75 mM KCl
10 mM DTT
0.25% (v/v) Igepal CA-630 (Sigma)
Store up to 12 months at –20°C

Tailing wash buffer

50 mM potassium phosphate, pH 7 (*APPENDIX 2*)
1 mM DTT
0.25% (v/v) Igepal CA-630 (Sigma)
Store up to 12 months at –20°C

Tween 20 wash buffer

50 mM Tris·Cl, pH 8 (*APPENDIX 2*)
75 mM KCl
10 mM DTT
0.5% (v/v) Tween 20
Store up to 12 months at –20°C

COMMENTARY

Background Information

Overview of amplification methods for small amounts of mRNA

With the completion of the human genome project and the introduction of technologies such as DNA microarrays and laser microdissection, many fields in biology and medicine await the application of comprehensive gene expression analyses of specific cell types isolated from defined tissues. The first protocols for the amplification of single cell mRNA were introduced in the late 1980s and early 1990s (Belyavsky et al., 1989; Brady and Iscove, 1993) and their development as well as their technical differences and application have been recently reviewed (Brady, 2000). All protocols are based on either of two principal approaches—linear amplification by T7 RNA polymerase or PCR amplification. Both procedures have advantages and disadvantages, and the one used depends on the experimental situation.

As a general rule, PCR-based methods are easier to handle and less time consuming, although there are concerns about the quantitative reliability of measurements obtained after exponential amplification (Brail et al., 1999). The linear amplification achieved by T7 RNA polymerase, also referred to as the Eberwine protocol (Eberwine et al., 1992; Kacharmina et al., 1999), has the advantage that a failure to amplify a given transcript will not be exponentially transmitted. On the other hand, there are several publications using PCR-based protocols showing that the relative abundance of transcripts is preserved even after 50 cycles. T7 RNA polymerase–based methods have been applied to cDNA and oligonucleotide arrays, but so far, the least number of cells that could be used successfully was ~1000 (Luo et al., 1999).

The methods provided in this unit are PCR approaches, and therefore are inherently prone to exponentially propagate initial amplification errors. The authors' primary intention was to obtain a qualitative representation of a single-cell transcriptome rather than preserving the numerical ratios of transcript abundance (Klein et al., 2002). Having established the method for single cells, the authors saw that quantitative differential analysis of gene expression with higher cell numbers (100 to 1000 cells) works quite well (Zohlnhofer et al., 2001a,b). This

seems to result from the fact that all experimental steps were optimized individually and in combination, that the number of steps was kept to a minimum, which led to high-complexity transcriptomes when the amplicons derived from single cells were hybridized onto cDNA arrays. Three points seemed to be particularly important. First, random primers reduce the length of primary transcript and enable subsequent amplification within the optimal range for PCR. Second, a poly-G tail provides a much better primer binding site than a poly A or poly T tail. Third, a poly-C containing PCR primer (binding to the poly G tail) should not be combined with any other primer sequence. Therefore, the flanking region of the cDNA synthesis primer has to be a poly-C track and a single PCR primer is used. A high annealing temperature and the addition of 3% formamide provide highly specific and optimal conditions for such sequences.

Reproducibility on a single-cell level is very difficult to assess, as two individual cells cannot be assumed to be identical and in the same functional stage. To exclude intercellular variation, the cDNA of an individual cell was divided prior to amplification, and then the variation of the resulting expression patterns (which was presumably introduced by the different methodological steps) was tested. Although random priming during cDNA synthesis, labeling, and hybridization add to the total variation, overall congruence of the two halves from one cell after global PCR was remarkably high for strong and intermediate signals. The weaker the signal, the more likely it was lost in one of the two halves (Klein et al., 2002). Therefore, when single cells are analyzed, the lack of a signal is more difficult to interpret and the authors recommend using independent methods such as real-time PCR or antibody staining.

Oligo arrays have become increasingly available from commercial suppliers (Affymetrix, Clontech, Qiagen, MWG-Biotech). Most of the sequences on these arrays are selected from the 3′ end of a transcript. In those cases where the 5′–3′ ratio is included into the bioinformatic evaluation (Affymetrix), one should not include the random primers, as the ratio will be shifted to the 5′ end. Here, initial results indicate that the $CFl5CT_{(24)}$ alone results in more quantitative results (if using the Affymetrix system, do not forget to include the T7 promoter into the oligo in the order: 5′-poly-C-flank, T7 promoter, $dT_{(24)}$-3′). In addition, if enough cells are available that allow division of the sample, it is advisable to determine the number of cycles needed to reach the plateau of the PCR reaction. Quantification is more precise during the linear phase of PCR, i.e., just before the plateau is reached. This can be done by setting up and running the PCR with half of the cDNA, and then running a gel of 3-µl aliquots that are taken during the PCR at various cycle numbers between 20 and 40 cycles (e.g., cycle 20, 24, 28, etc.). Then, the PCR may be set up with the other half of the cDNA, programming the thermal cycler for the ideal number of cycles.

Critical Parameters

For best results, adhere to the following rules.

High-quality enzymes are critical for amplification success. In particular, terminal deoxynucleotide transferase (TdT) and RNaseH– deficient reverse transcriptase (RT) need to be selected carefully. TdT is delivered either in cacodylate-containing or KH_2PO_4-containing storage buffers. Avoid cacodylate-containing buffers unless they can be highly diluted. Reverse transcriptase is sometimes contaminated with bacterial DNA. Therefore, check different batches of a manufacturer.

Always work under sterile conditions with filter tips and avoid RNase contamination. It is also of great importance to protect the reactions from any nucleic acid contamination because DNA/RNA molecules present in the tube will be amplified as well (reverse transcriptase also uses DNA as a template).

Always work on ice.

During all wash steps using the magnet, check that no beads are aspirated with the supernatant.

Do not allow the beads to dry out. This preserves the binding of the mRNA to the beads.

Working with more than eight samples at once is not recommended, since it increases the duration of the procedure and consequently favors RNA degradation.

Clumped beads typically result from genomic DNA. Refer to Table 25B.8.1 to adjust the bead volume to the cell number.

Perform the hot-start procedure quickly, since keeping a single-stranded cDNA at 78°C for extended times can destroy the template.

Troubleshooting

Global amplification

There is no way to check the individual steps prior to PCR amplification. Before hybridizing a sample to an array, test amplification success

Table 25B.8.6 General Troubleshooting Guide

Cause/problem	Possible solution
Negative PCRs or no primary product	
Inactive reagents	Because all steps are critical, be sure that all reagents have been properly stored and handled. Primers should be dispensed into aliquots prior to use in order to prevent repeated freeze/thaw cycles; do not store diluted dNTP or dGTP too long; check expiration dates of enzymes; check tailing buffer and TdT storage buffer for absence of cacodylate; formamide must be deionized. High-quality enzymes and primers are most essential.
Gene-specific PCR	Most gene-specific PCRs will work with the primary PCR products as template, but be prepared to re-test the annealing temperature for the CP2-amplified cDNA. Sometimes, gene-specific PCR works better on 1:10 to 1:1000 diluted template than on undiluted amplicons.
No or weak signals on cDNA arrays	
Degraded Dig-UTP	Digoxigenin is alkali-labile. Therefore, check pH of all solutions after hybridization.
Film exposure	Be sure to expose the hybridized/exposed side of the filter. Re-expose cDNA array, prolong exposure time, correct orientation of coated film.
Hybridization temperature	Control the hybridization temperature. Some hybridization buffers work at 68°C, others at 45°C, depending on the content of DNA-denaturing substances.
Denaturation of DNA	Both probe and target have to be single stranded. Check denaturation and the protocol for array preparation.
Suspiciously identical results with different probes on cDNA arrays	
Co-amplification, labeling, and hybridization of bacterial/plasmid DNA with cellular cDNA	Control the quality of the array by hybridizing labeled *E. coli* and plasmid DNA to the array; use arrays of which the cDNAs have been amplified by insert-specific PCR or oligonucleotide arrays
	Check for possible sources of contamination in the sample; test different batches of reverse transcriptase
	If contamination is unavoidable, label the negative control and add increasing amounts of blocking DNA (i.e., *E. coli* or DNA of the most frequently used plasmids used to generate the array) until the filters are clean
High background of cDNA arrays	
Probe concentration	Check concentration of added probe. Concentrations >1.5 µg/ml can result in high background
Addition of probe	Never add undiluted probe to the array. Direct contact with the nylon membrane will result in dark areas/spots. Dilute the labeled probe in ~1 ml hybridization buffer and be careful not to pour it directly onto the filter.
Restringency washes	Unbound or unspecifically bound probe must be entirely washed out. Check SSC concentrations and washing temperatures.
Alkaline phosphatase	Alkaline phosphatase is expressed by bacteria. Check/autoclave buffers used for developing the filters.
Filters	Nylon membranes can be stripped and re-hybridized up to six to eight times. Repeated use, however, will increase background every time.
Precipitated Fab fragments, degraded anti-digoxigenin-alkaline phosphatase	Spin down antibody solution prior to use and use the supernatant only

by gene-specific PCRs for housekeeping genes and one less abundant, but more or less consistently expressed, gene of the investigated cells. In addition, it is advisable to run a gel with 5 µl of the primary PCR. It should show a smear ranging from 200 to 2000 bp without bands. A sample without the addition of cellular mRNA should also be checked since contamination can be detected this way. If a smear (originating from DNA contamination in the enzyme preparations) is at all present, in the negative control, it should be smaller (100 to 300 bp) and less intense. Note that individual bands sometimes result from concatamerization of the primers and do not necessarily indicate contamination.

Gene expression analysis on nylon arrays

Roche Diagnostics provides an excellent manual with the digoxigenin hybridization kit. All relevant information for non-radioactive array analysis can be found there.

A general troubleshooting guide is presented in Table 25B.8.6.

Anticipated Results

After PCR amplification, the DNA content of the sample should be measured by following the Support Protocol in *UNIT 2.6* or by optical density, an ethidium bromide plate compared with a standard, or alternative methods like Nucleic dotMetric (Genotech). The anticipated amount of cDNA is between 100 and 300 ng/µl.

Before hybridization, amplification success is tested by checking the primary product and by gene-specific PCR as described. Running a gel with the primary PCR product, a smear ranging from 100 to 2000 bp without bands should be observed. Using single cells sometimes results in a smaller range. A sample without cellular mRNA should be included throughout the whole experiment as a negative control. From this sample, there should be no apparent smear; however, sometimes smears can be observed when the reagents, especially enzyme preparations, contain nucleic acids. Controlling the primary amplification by gene-specific PCRs for two housekeeping genes and one constantly but less abundantly expressed gene is recommended. The negative control must be negative for all gene-specific PCRs. Gene-expression analysis on nylon arrays should result in films with low background and ~20% to 40% positive hybridization signals for >10 to 20 cells. Positive signals from single cells should range from 5% to 25% of spotted cDNAs, depending on activation stage. The housekeeping genes spotted on each filter should yield strong positive signals. The negative-control spots show no signal unless sample and array are contaminated with bacterial/plasmid-derived DNA.

Time Considerations

Global amplification of cellular cDNA

The time needed depends on the incubation/reaction times and the number of samples (washing >7 samples using the magnet is time consuming). It takes ~45 to 60 min for cell lysis, mRNA capture to the beads, and washing steps. At this point, the mRNA on the beads can be frozen and stored at −80°C. The subsequent cDNA synthesis, tailing reaction, and PCR amplification must be performed without interruption. cDNA synthesis including the wash steps will take ~1.5 hr and the tailing reaction will take an additional 1.5 hr. The PCR will take 3 to 4 hr and can be run overnight.

Non-radioactive gene expression analysis on nylon arrays

It takes ~30 min to set up the labeling PCR and the PCR itself will take ~1.5 hr. Pre-hybridization of samples requires at least 6 hr when cDNA arrays are used. Hybridize the labeled probe over 2 nights when few cells were used; cDNA from higher cell numbers might be hybridized for 1 night. Non-radioactive development of filters will require ~3 hr. The exposure time of the film has to be individually evaluated, but usually two films developed at 15 and 60 min are sufficient.

Data analysis of hybridized cDNA arrays

Scanning of the films will take ~10 min per film and data analysis by array vision will take 30 to 60 min per film.

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CHAPTER 25

Discovery and Analysis of Differentially Expressed Genes in Single Cells and Cell Populations

INTRODUCTION

For decades, molecular biologists have been discovering and analyzing genes that are differentially expressed. Initially, discovery and analysis were achieved one gene at a time. This was followed by cDNA cloning methods to identify genes that were expressed in a given tissue. However, this left the investigator with a large number of genes to screen for differential expression. A major advance was the development of subtractive cloning in the 1980s, which greatly enriched for genes that were expressed in one cell or tissue type rather than another. Since the advent of PCR using thermostable DNA polymerases in the late 1980s, older methods have been refined and many new techniques have been developed that make discovery of differentially expressed genes much more facile and permit the analysis of differential gene expression at the single cell level.

This chapter consists of protocols—some of them older, some of them newer—for two kinds of methods. The first of these are amplification-based methods for analysis of individual cells and are contained within Section 25A. *UNITS 25A.1 & 25A.3* describe the use of laser-capture microdissection (LCM) of histological specimens so that one can analyze nucleic acids, in individual cells, using PCR or other methods. The LCM protocols in *UNIT 25A.1* are optimized for analysis of animal cells and tissues, while those in *UNIT 25A.3* are optimized for plant cells and tissues. Additionally, *UNIT 25A.3* describes a protocol for in vitro transcriptional amplification of RNA, which is a frequently used alternative to PCR that entails linear rather than exponential amplification, and thus has certain advantages (and disadvantages) relative to PCR. *UNIT 25A.2* describes methods for fixation of tissues and subsequent dissociation of the fixed tissue into single cells whose nucleic acids can be analyzed by PCR-based or other methods.

Section 25B contains molecular methods for discovery of differentially expressed genes. *UNIT 25B.1* (formerly *UNIT 5.8B*) describes production of a subtracted cDNA library while *UNIT 25B.2* (formerly *UNIT 5.9*) describes the refinement of PCR-based subtractive cDNA cloning with a support protocol for slot blot hybridization to monitor sublibraries. Subtracted cDNA libraries provide a method where cDNAs are synthesized from mRNA from the desired tissue or cell type and then sequences that are also expressed in a control tissue or cell type are removed by hybridization and selection.

UNIT 25B.3 describes a powerful application of PCR to gene discovery, differential display. This technique allows the identification and subsequent isolation of differentially expressed genes that requires no knowledge of sequences, but rather PCR amplification using arbitrary oligonucleotides and high-resolution polyacrylamide gel electrophoresis. *UNITS 25B.4 & 25B.5* describe variations on differential display, restriction-mediated differential display (RMDD), and amplified fragment length polymorphism (AFLP) based transcript profiling, which make use offrequently cutting restriction enzyme sites in cDNAs and may offer advantages to the practitioner.

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UNITS 25B.6 & 25B.7 contain different PCR-based approaches for determining what genes are expressed in a given cell or tissue type. *UNIT 25B.6* describes serial analysis of gene expression (SAGE). This technique generates concatemers of short cDNA sequence tags that have been ligated together. These concatemers can be cloned, sequenced, and analyzed with the aid of specialized software to identify differentially expressed genes and to compare their expression with those present in other SAGE libraries. The unit also contains a protocol for cloning cDNA starting with a given sequence tag. *UNIT 25B.7* describes representational difference analysis (RDA). RDA combines PCR-mediated kinetic enrichment with subtractive hybridization to generate 0.2 to 2 kbp sequences that are distinct to genomic DNA or mRNA in one cell type versus another. These can then be cloned and sequenced or otherwise analyzed.

UNIT 25B.8 describes a protocol in which both PCR and reverse transcription have been optimized to permit the detection and semi-quantitative analysis of transcripts from single cells, small tissue biopsies, and microdissected samples. These protocols extend and complement those provided in *UNITS 25A.1, 25A.2, & 25A.3*.

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